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# Redistribution of Minerals in Apple Fruit during Storage: Effects of Storage Atmosphere on Magnesium and Phosphorus Concentrations

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*ABSTRACT*

*The median transverse redistribution of magnesium and phosphorus in Cox's Orange Pippin apples stored at 2·8°C or 4°C was investigated. The more detailed investigation at 4°C showed that phosphorus accumulated in the core zone throughout storage in air, but that the inward movement of magnesium was delayed in the early weeks when magnesium concentration increased in the peel. Storage atmospheres of 2 or 1·25% oxygen (<1% carbon dioxide) inhibited movement of both elements to the core zone and, for a few weeks following the establishment of a 1·25% oxygen atmosphere, magnesium was withdrawn from the core zone. An inward shift of magnesium from the peel to the inner cortex occurred several weeks prior to the development of visible low temperature breakdown symptoms in apples stored in 1·25% oxygen.*

*Similarities between the effects of low oxygen and low temperature storage on the redistribution of magnesium and phosphorus and on the induction of low temperature breakdown are discussed.*

*Key words*: Apple, fruit, magnesium, phosphorus, storage, oxygen, low temperature breakdown.

## 1 INTRODUCTION

Changes in the distribution of minerals within fruit have been investigated to clarify their role in promoting or suppressing various physiological disorders during the storage of apples. For extended marketing (Sharples 1982), Cox's



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Orange Pippin apples are stored commercially in controlled atmospheres (CA) in which oxygen concentrations are maintained at 2 or 1·25%, with the carbon dioxide concentration kept below 1%. One limitation of this technique is that these very low concentrations of oxygen increase the risk of low temperature breakdown (LTB) developing (Wilkinson and Fidler 1973; Sharples 1982; Sharples and Johnson 1982), even at recommended storage temperatures (ie *c* 4°C). The resistance of Cox apples to LTB during their storage in air has been associated with high concentrations of magnesium and phosphorus in the fruit (Perring 1968).

The transverse redistribution of these elements within Cox apples during storage in air is altered when the temperature is lowered sufficiently to induce LTB (Perring 1985; Perring and Pearson 1986). In a preliminary investigation of the redistribution of magnesium and phosphorus in median transverse slices of Cox apples stored in air at 2·8°C, migration of both elements to the core zones appeared to be inhibited by CA storage. Subsequent measurements of magnesium and phosphorus redistribution, reported here, were made to gain further information on the effects of CA storage.

## 2 MATERIALS AND METHODS

### 2.1 Fruit

Cox's Orange Pippin apples were used for both experiments. In 1980 apples were obtained from two commercial orchards (A and B) selected (using data from a series of fruitlet analyses) to provide two fruit samples of similar average mass with different concentrations of both magnesium and phosphorus. The fruit was picked from orchards A and B on 16 and 17 September, respectively (ie before the main harvest of 19 September: Table 1), sorted (to remove blemished or exceptionally large and small apples) and divided into sub-samples of 20 apples. No calcium or fungicide drenches were applied. Sub-samples were stored at 2·8°C in

**TABLE 1**

Mean Mass (g) and Composition (Excluding Seeds and Stalks) of Bulked Samples of Whole Cox's Orange Pippin Apples (mg 100 $g^{-1}$ fresh weight)

| | *Sample source* | | |
|---|---|---|---|
| | *A* | *B* | *C* |
| Harvest date | 19 September 1980 | 19 September 1980 | 21 September 1984 |
| Total number of apples, *N* | 180 | 180 | 2160 |
| Number of sub-samples,[a] *n* | 9 | 9 | 72 |
| Mean mass+SE[b] | 104±2·06 | 104±1·35 | 110±0·36 |
| Number of apples analysed | 30 | 30 | 90 |
| Mg | 6·2 | 5·5 | 4·3 |
| P | 15·0 | 10·4 | 8·9 |

[a]Each of *N*/*n* apples.

[b]SE, with *n*-1 df calculated from the mean mass of apples in each sub-sample.

air or in 2 or 1·25% oxygen with carbon dioxide kept below 0·5%. Duplicated 'initial' sub-samples from orchard A were sectioned immediately after harvest but those from orchard B were stored at 2·8°C until they could be sectioned on 23 and 26 September. Duplicated sub-samples were removed from the CA stores on 17 March 1981 and held at 2·8°C until they were sectioned (17–19 March). Sub-samples from both sites were removed from air storage for sectioning at intervals in 1981 until 20 March.

In 1984, the fruit was picked from trees (on MM.106 rootstock) of an experimental orchard (C) on 20 September. Sub-samples of 30 apples were stored at 4°C either in air or in a commercial CA store (1·25% oxygen, <0·3% carbon dioxide). Full details of sub-sampling, storage and sectioning were described previously (Perring and Pearson 1987).

### 2.2 Sectioning

All sub-samples were washed in running distilled water and allowed to dry naturally prior to sectioning. Plugs representing a median transverse slice were cut from each apple with a 12 mm-diameter cork borer and sectioned into five zones, as described previously (Perring and Pearson 1984, 1987). Discs from equivalent zones of all the apples in a sub-sample (seeds were removed from the core zone sections) were frozen together in liquid nitrogen, stored at −20°C and subsequently grated (Perring and Pearson 1984, 1987).

Seeds and stalks were removed from the sub-samples taken for analyses of the whole fruit. In 1984 these apples were frozen at −20°C and subsequently grated at −15°C. Seeds and stalks were also removed from the samples of whole apples taken in 1980 before they were homogenised prior to blending with water and sub-sampling for analysis (Holland *et al* 1975).

### 2.3 Ashing and analyses

Sub-samples of 2 to 5 g of frozen, grated material were ashed in platinum crucibles, the ashes were dissolved in hydrochloric acid, and magnesium was determined by flame absorption spectrophotometry (Perring 1974). Phosphorus was measured colorimetrically (Fogg and Wilkinson 1958). The digestion and analytical procedures used for the 1980 samples of whole apples have been described elsewhere (Holland *et al* 1975).

### 2.4 Processing results

Concentrations of the elements were measured on a fresh weight basis.

Standard errors of differences between means were derived from data for replicated sub-samples as follows: in 1980–81 pooled data from the duplicated initial sub-samples and the CA-stored sub-samples were used; in 1984–85 the pooled data of the triplicated initial and all the triplicated air- and CA-stored sub-samples were used.

### 2.5 Low temperature breakdown assessment

Low temperature breakdown (LTB; Wilkinson and Fidler 1973) developed in some of the CA-stored apples. As symptoms were either very slight (immediately

adjacent to the vascular bundles in the inner cortical zone) or slight (a more diffuse browning of the mid-cortex), percentages of apples affected rather than indices of incidence×severity (Perring 1985; Perring and Pearson 1986) were recorded.

## 3 RESULTS

Mean masses per apple at harvest and average concentrations of magnesium and phosphorus in the samples used in both years are given in Table 1. In 1980–81, the stored apples had lost 3 to 4% of their initial mass by the end of the experiment. In 1984, apples from either store lost 4% of their initial mass over the course of the experiment.

### 3.1 Storage in 1980–81

Figure 1 shows the concentrations of magnesium in the various zones of median transverse slices of apples from sites A and B in 1980–81. Large increases in concentrations occurred in the core zones of fruit from both sources during 110 days of air storage. Such changes were not evident in three of the samples stored in CA conditions for 180 days. The increase in the concentration of magnesium in the core zone of apples from the fourth sample (from site A, stored in 1·25% oxygen) was smaller than in air storage. The concentration of magnesium also increased in the peel during 110 days of air storage, especially in that of fruit from site B, but had apparently changed little or even declined slightly after CA storage. CA conditions also apparently prevented the decline in the concentration of magnesium in the mid-cortex that occurred during the storage of apples from both sites in air.

Concentrations of phosphorus in the same zones are shown in Fig 2. The increases in the concentrations of phosphorus in the core zones of apples from both sites during storage in air were similar to those of magnesium, ie 3 to 4 mg 100 $g^{-1}$. Storage in CA also apparently reduced the transfer of phosphorus to the core zone and usually reduced the concentration of phosphorus in the peel. Storage in air affected phosphorus in the peel of apples from the two sites differently. In the apples with the higher overall level of phosphorus (site A, Table 1), the phosphorus concentration in the peel declined during 110 days' storage whereas that in the peel of fruit from site B increased.

### 3.2 Storage in 1984–85

Differences between the long-term effects of storage in CA and air in 1984–85 (Fig 3) were broadly similar to those in 1980–81. However, the detailed data show that the differences in the concentration of magnesium in the core zone developed early during storage. This was because of the rapid decline in concentration of magnesium in the cores of CA-stored apples after an oxygen concentration of 1·25% was established (ie after 2 weeks' storage). The concentration of magnesium in the cores of the air-stored apples remained fairly constant during these early weeks and then increased steadily.

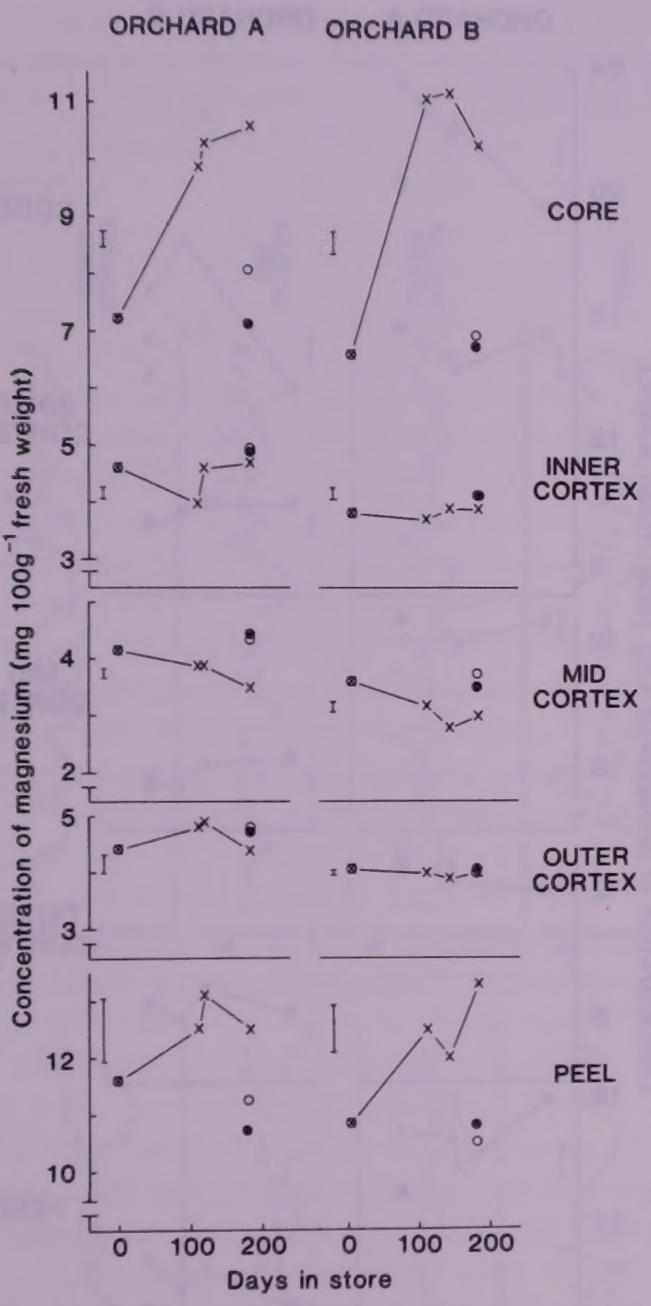


**Fig 1.** Samples A and B, 1980–81. Effects of low oxygen storage at 2·8°C on the final distribution of magnesium across median transverse slices of apples from two orchards (A and B) compared with changes during air storage at 2·8°C. Means for duplicated subsamples in 2% oxygen=●, in 1·25% oxygen=○, initially=⊗; single sub-samples in air=×. SEDs are shown as bars to the left.

Differences in the concentration of phosphorus in the core developed over a longer period. There was a gradual decline in the concentration of phosphorus in the CA-stored fruit, after a brief initial rise, whereas that in the air-stored fruit generally continued to increase.

The concentration of magnesium in the peel of the air-stored fruit increased over the first 56 days' storage, declined and then increased again towards the end of storage. A similar initial rise occurred in the peel of Cox's Orange Pippin apples stored at 3·8°C in 1979 (not shown here) but to a lesser extent and over a shorter period (30 days). The somewhat different pattern of changes in the concentrations

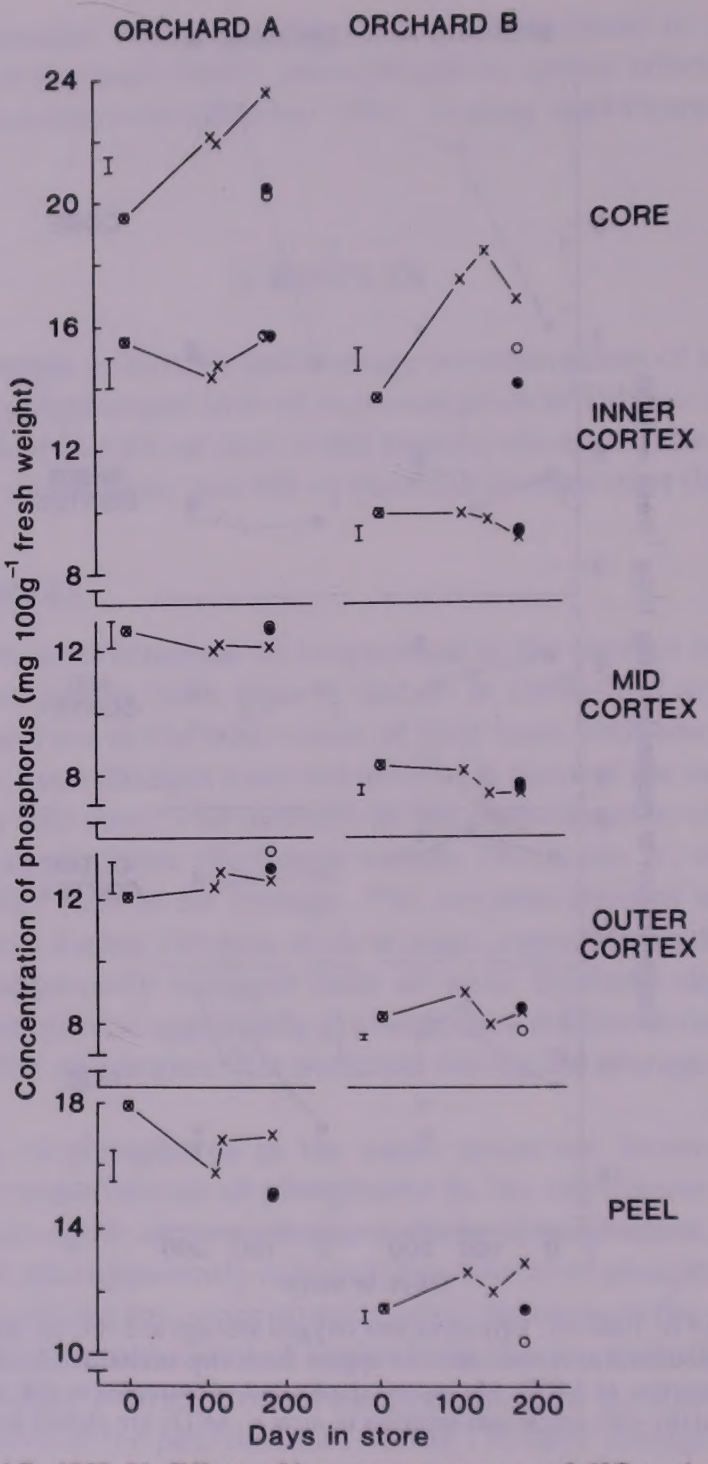


**Fig 2.** Samples A and B, 1980–81. Effects of low oxygen storage at 2·8°C on the final distribution of phosphorus across median transverse slices of apples from two orchards (A and B) compared with changes during air storage at 2·8°C. Symbols and SEDs as in Fig 1.

of both magnesium and phosphorus in the peel of fruit from the two stores resulted in statistically significant differences in the effects of storage atmosphere at various times during storage. The concentrations of both elements became consistently lower in the peel of the CA-stored fruit during the final 30 days in store.

Changes in distribution of both elements during storage (ie the proportion in each zone of the total amounts of each element in the whole slice) generally

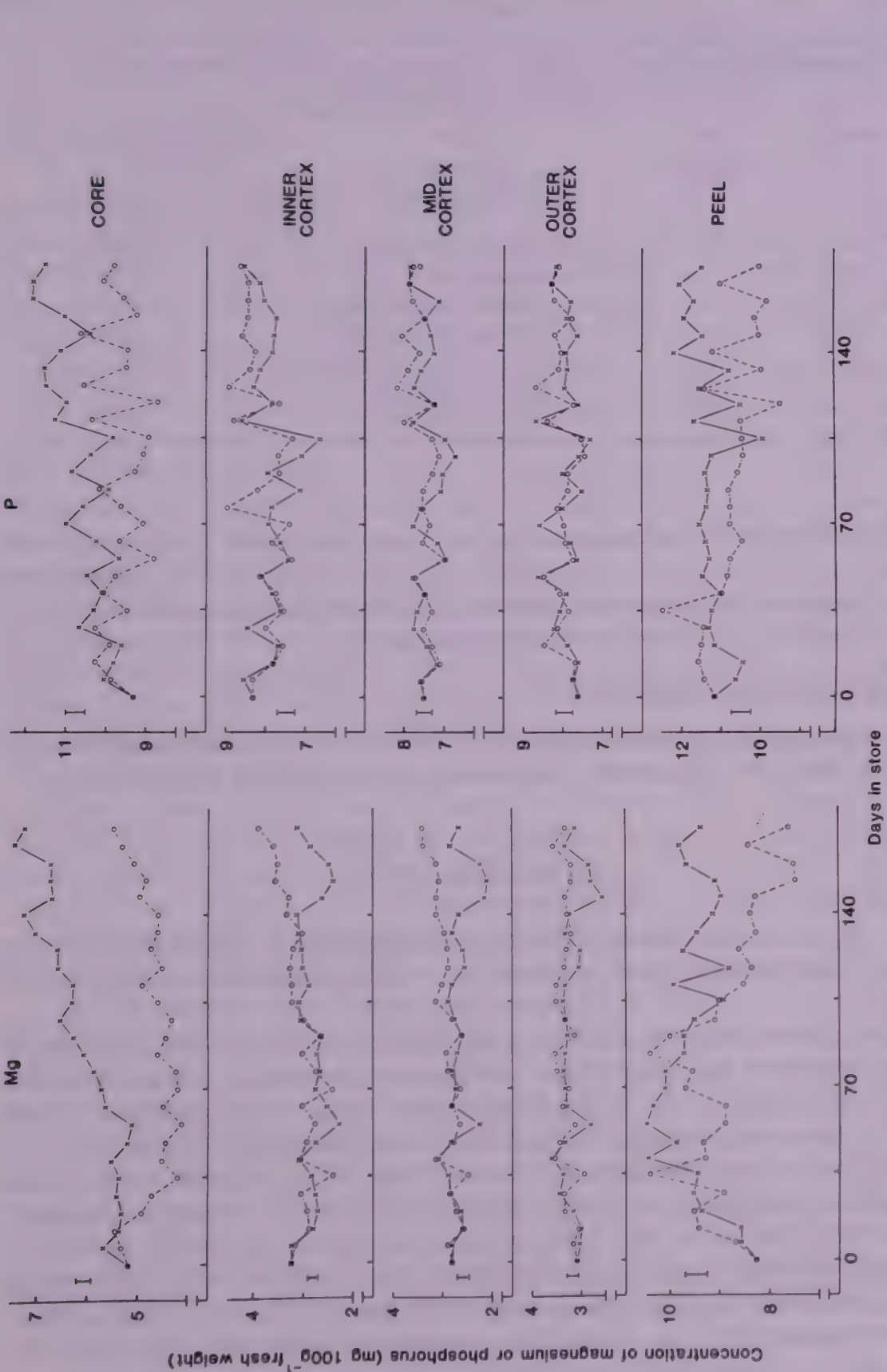


**Fig 3.** Sample C, 1984–85. Effects of storage in 1·25% oxygen on the distribution of magnesium and phosphorus across median transverse slices of apples stored at 4°C compared with changes during air storage at 4°C. Means for triplicated sub-samples in 1·25% oxygen=○, in air=×. SEDs are shown as bars to the left.

**TABLE 2**
Percentages of Apples Stored in Low Oxygen Atmospheres Affected by Low Temperature Breakdown (LTB) when Sectioned in 1981 and during the Final 28 Days' Storage in 1985

| *Sample* | *Storage temperature (°C)* | *Date examined (days in store)* | *Percentage affected by LTB* | |
|---|---|---|---|---|
| | | | *2% $O_2$* | *1·25% $O_2$* |
| A | 2·8 | 19 March 1981 (184) | 0·0 | 15·0 |
| B | 2·8 | 19 March 1981 (183) | 0·0 | 17·5 |
| C | 4·0 | 14 February 1985 (146) | — | 0·0 |
| C | 4·0 | 20 February 1985 (152) | — | 0·0 |
| C | 4·0 | 28 February 1985 (160) | — | 14·3 |
| C | 4·0 | 7 March 1985 (167) | — | 24·1 |
| C | 4·0 | 14 March 1985 (174) | — | 46·7 |

corresponded to those in concentration, with two exceptions: the proportion of magnesium in the core zone of the air-stored fruit declined over the first 40 days; and the increases in the proportion of magnesium in the peel of the CA-stored fruit at 35 and 84 days were less clearly defined.

### 3.3 Low temperature breakdown

The percentages of apples affected by LTB in fruit stored in CA conditions are given in Table 2. No symptoms were seen in apples stored in air in either year.

## 4 DISCUSSION

In 1980–81, the lower concentrations of magnesium (Fig 1) and phosphorus (Fig 2) in the core at the end of the experiment were clearly attributable to the deficit of oxygen.

The comprehensive data obtained in 1984–85 showed that the deficit of oxygen not only inhibited the redistribution of magnesium and phosphorus but also altered it. The effect of CA storage on magnesium redistribution paralleled that of storage at low temperature (in the period prior to the onset of LTB symptoms) in an earlier experiment (Perring and Pearson 1986). Thus in the first 80 days the proportion of magnesium in the core zone was reduced by storage in CA conditions at 4°C or in air at 0°C compared with storage in air at 4°C and 3·5°C, respectively. Similar trends for phosphorus were evident but less definite in both experiments. The results contrast strongly with those obtained for calcium (Perring and Pearson 1987): the general redistribution of calcium from the core to the outer zones was similar in CA- and air-stored fruit from orchard C in 1984.

LTB is caused by the subjection of apples to low temperatures (Wilkinson and Fidler 1973) or atmospheres high in carbon dioxide or humidity (Wilkinson and Fidler 1973) or low in oxygen (Sharples 1982; Sharples and Johnson 1982) which influence metabolic changes or the physical characteristics of the fruit. The development of the disorder is retarded (Perring, 1968), but not prevented, by

high overall concentrations of magnesium and phosphorus in fruit of a susceptible variety. In the investigation reported here, CA storage induced LTB in more apples of sample C, which had very low overall concentrations of both elements (Table 1), than in fruit of samples A and B by mid-March (Table 2) despite storage at a higher temperature.

The reversal or retardation of the translocation of magnesium and phosphorus to the core zone by either low temperature or low oxygen concentration appears to be linked to the predisposition of Cox apples to LTB. The requirement of the core for both elements may be high because at harvest this zone always has higher concentrations of both elements than the surrounding flesh (Perring and Pearson 1984). Spartan apples, which are resistant to LTB even when stored in an atmosphere of 2% oxygen at temperatures as low as −1·0°C (Stow 1986), have lower overall concentrations of magnesium and phosphorus in the fruit than Cox apples. However, they have higher proportions of both elements in the core zone than do Cox fruit (Perring and Pearson 1984) and continue to accumulate magnesium and especially phosphorus in the core zone during storage in atmospheres low in oxygen (Perring 1984) and/or at low temperatures (Perring 1984; Perring and Pearson 1986).

In trials designed to increase the phosphorus content of apples, sprays of phosphates, applied in the orchard, reduced the severity of LTB in Cox apples (Johnson and Yogaratnam 1978) and the incidence of LTB in Bramley's Seedling apples (Yogaratnam and Sharples 1982). In both trials the concentration of magnesium, in addition to that of phosphorus, was often increased in the whole fruit, particularly in Cox apples. Although it was argued that the effect on magnesium concentration was too small to influence LTB (Yogaratnam and Sharples 1982), a small increase in concentration could have had a disproportionate effect if it were concentrated in the core zone. It was also suggested (Johnson and Yogaratnam 1978; Yogaratnam and Sharples 1982) that phosphate sprays applied early (ie during the time of rapid cell division in the fruit) were more effective in reducing LTB incidences than sprays applied later because of possible effects on cell division and membrane lipids. However, it is in this same early period that the core zone of the apple develops much more rapidly than the cortex and hence phosphorus and magnesium concentrations in the core zone are more likely to be increased by phosphate sprays.

Magnesium is usually transported into damaged tissues (Perring 1985; Perring and Pearson 1986). In earlier experiments with air-stored apples, a large inward shift of magnesium from the peel coincided with the appearance of visible LTB symptoms. A similar, more gradual, shift to the mid-cortex and then the inner cortex of the CA-stored fruit preceded the development of visible symptoms there in 1985 (Fig 3, Table 2). This suggests that in CA conditions tissue damage may develop several weeks before browning occurs.

## 5 CONCLUSIONS

These investigations have demonstrated large effects of low oxygen storage atmospheres on the redistribution of both magnesium and phosphorus within Cox

apples. They also show the value of sampling successively at short intervals. If the link between the ability of apples to continue translocating phosphorus and/or magnesium to the core zone, despite CA conditions or low temperature storage, and reductions in incidences of LTB can be established, a simple test (based on analyses of core zone tissue only) could be used to screen new apple varieties for LTB resistance.

Further research should examine biochemical and biophysical changes involved in the redistribution of minerals and biochemical processes affected by extra supplies or withdrawals of phosphorus and magnesium. In particular, biochemical changes taking place in the zones often discarded by research workers should be investigated, ie in the inner cortex and core, especially in relation to the onset of LTB, and in the peel where the requirement for magnesium apparently alters throughout storage.

## ACKNOWLEDGEMENTS

The help of the Directors and staff of Ace Fruit Ltd, Galleywood, Chelmsford, with CA storage in 1984–85 and the removal of sub-samples at weekly intervals is gratefully acknowledged. The CA samples in 1980–81 were stored by Mr D S Johnson. The 1980–81 samples of whole apples were analysed at the MAFF/ADAS sub-centre, Wye, Kent, as part of a concurrent joint survey experiment. Mr K J Martin and Dr D A Preece provided statistical advice.

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# A Time-course Study of Glucosinolates in the Ontogeny of Forage Rape (*Brassica napus* L)

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*ABSTRACT*

*One cultivar and two breeding lines of forage rape, grown in a glasshouse, were sampled at weekly intervals from 9 to 22 weeks after sowing. Separate determinations of glucosinolate content were made on leaves and stems by HPLC. There were differences in the proportion and concentration of individual glucosinolates between the rapes and between leaves and stems, showing that levels of individual glucosinolates cannot be predicted from total glucosinolate content. Of particular interest was the variation with harvest date in the levels of individual glucosinolates. This has considerable implications for the comparison of levels of individual glucosinolates between cultivars and crops, and suggests that detailed agronomic field studies are required to determine whether the intake by animals of potentially toxic glucosinolates, in particular progoitrin, can be minimised by exploiting these seasonal differences in glucosinolate concentrations. Progress has been made in lowering the level of this glucosinolate, and the possibility of breeding forage rape with very low or zero levels of progoitrin is indicated.*

*Key words*: Glucosinolates, brassicas, forage rape, thiocyanate ion, progoitrin.

## 1 INTRODUCTION

The existence of goitrogenic factors in the brassicas has been recognised since 1928 following experiments on the feeding of high levels of cabbage (*Brassica oleracea*) which resulted in the development of goitre in rabbits (Chesney *et al* 1928). The condition was noted particularly in cattle fed on kale (*B oleracea*) and



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was attributed to high levels of thiocyanate ion produced in the rumen following the breakdown of indole glucosinolates (Paxman and Hill 1974; Forss and Barry 1983). From the work of other fodder brassica breeders (Ellerström and Josefsson 1967; Josefsson *et al* 1972; Johnston 1980), for practical breeding purposes at the Scottish Crop Research Institute (SCRI) forage rape (*B napus* var *biennis*) was assumed to be similar to kale, in terms of both the distribution and the quantity of individual glucosinolates. However, the results of analyses of freeze-dried samples of forage rape over a 5-year period at SCRI (unpublished) showed that thiocyanate ion was present at only between 10 and 15% of the level found in kale. Other workers (Paxman and Hill 1974; Bradshaw *et al* 1983) quantified the level of thiocyanate ion in *B napus* at 20 to 30% of that found in *B oleracea*. More recently, it has been shown that another glucosinolate, 2-hydroxybut-3-enyl glucosinolate, commonly known as progoitrin and the precursor of 5-vinyloxazolidine-2-thione (goitrin), is present at particularly high levels in the stem and leaves of forage rape (Bradshaw *et al* 1983, 1984). Progoitrin is also the major glucosinolate present in the seed of oilseed rape.

As yet, little is known about the metabolism and physiological effects in sheep of the glucosinolates, so breeding lines with both low and high levels of progoitrin and other glucosinolates are being bred at SCRI for use in subsequent feeding experiments. However, it would be useful to know if there are particular stages in the growth of forage rape when progoitrin and other glucosinolates are likely to be present at high or low levels. In addition, it is now appropriate to examine any such variation between the different breeding lines of forage rape available at SCRI.

## 2 EXPERIMENTAL

### 2.1 Cultivars and breeding lines

For a preliminary experiment, only the cultivar Hobson, a giant-type rape, was used. In the main experiment, one cultivar and two breeding lines of forage rape were selected: (a) Hobson; (b) RS5 (recently named Bonar), *B napus* line resynthesised from thousand-head kale, curly kale and *B campestris* ssp *nipposinica*, crossed with the forage rape cultivars Nevin and Samo; and (c) 84411, a *B napus* resynthesised from Maris Kestrel kale, kohl-rabi and a clubroot-resistant stubble turnip.

### 2.2 Cultivation and harvesting

In the preliminary experiment, 30 seeds of Hobson were sown in Jiffy 7 pots on 27 April 1984. These were raised in a glasshouse at *c* 10°C without any additional heating or lighting. The plants were transferred to 15-cm pots on 12 June, using a peat/sand compost supplemented with inorganic nutrients, and moved to a glasshouse at 15°C. Plants were watered daily and fed once with John Innes Liquid Feed on 14 August. Ten randomly selected plants of similar height were harvested on 12 September, partitioned into leaves and stems, and immediately placed in a cold room at −20°C.

In the main experiment, sufficient seeds were sown in Jiffy 7 pots on 27 February 1985 to provide 170 uniform plants of each cultivar and breeding line, together with a surround for these so that edge effects could be avoided at sampling. These were raised as above, potted up on 2 April and moved to a controlled-environment glasshouse at 12°C. Over the period of the experiment the glasshouse temperature increased gradually to 18°C. The daily temperature fluctuation was ±3°C. Plants were watered by trickle irrigation and fed on 30 April, one month later and then at fortnightly intervals with John Innes Liquid Feed. One spray against powdery mildew (*Erysiphe cruciferarum*) was required on 6 June using calixin. Because of differences in height, plants of each cultivar or breeding line were kept in one block, separated from a neighbouring block by a 2-m gangway. The first harvest was on 25 April, nine weeks after sowing, and plants were then harvested at weekly intervals for the following 16 weeks. All harvest dates are given in the number of weeks from sowing. Ten plants were taken at random from within each block and, following measurements for a routine growth analysis, five of these were partitioned into leaves plus petioles, and stems, and immediately placed in a cold room at −20°C. Separate growth analyses on leaves and stems showed that the growth rates were uniform throughout the experiment. There were no significant differences in the rates of dry matter production for the leaves and stems of the three rapes used.

### 2.3 Preparation of plant material for chemical analysis

Once the samples were completely frozen, they were freeze-dried in a Millitorr S3921 vacuum freeze-drying unit for 72 h. The product was then milled through a 1-mm sieve in a Christy-Norris laboratory mill and stored in Sterilin plastic jars with screw tops at −20°C until analysis.

### 2.4 Extraction and chemical analysis

#### *2.4.1 Isolation and preparation of desulphoglucosinolates*

The intact glucosinolates were extracted by a procedure based on that described by Heaney and Fenwick (1980). Freeze-dried plant material (1 g) was suspended in 20 ml of boiling 70% v/v methanol and heated on a boiling water bath for 20 min. After filtration a further 20 ml of boiling 70% v/v methanol was added to the residue, and the suspension was heated for 5 min and again filtered. The filtrates from both extractions were combined, the methanol was removed by rotary evaporation *in vacuo* and the residual solution was made up to a final volume (10 ml) with distilled water.

A 2-ml aliquot was taken, 0·2 ml of 0·5 M barium and lead acetate solution was added and the resulting suspension was made up to 5 ml with distilled water. After centrifuging, 4 ml of the supernatant was applied to a 100-mg DEAE Sephadex A-25 (acetate form) column and desulphated overnight by the addition of aryl sulphatase (Sigma H-1). The desulphated glucosinolates were then eluted with 2 ml of distilled water.

#### *2.4.2 Separation of desulphoglucosinolates*

The desulphoglucosinolates were separated by high performance liquid chro-

matography (HPLC) using a Gilson dual pump chromatograph fitted with a 2 cm×2 mm id pre-column containing Lichoprep RP-18 (Anachem L) and a 25·0 cm×4·6 mm id Zorbax ODS (Du Pont Instruments Ltd) column. The temperature of the column was maintained at 30°C and the flow rate was set at 1·5 ml $min^{-1}$. The eluant was monitored at 230 nm (AUFS 0·05) and peak areas were integrated by a Shimadzu C-R3A Chromatopac programmed for area normalisation.

The elution profile used was broadly similar to that reported by Spinks *et al* (1984) and employed a two-solvent system consisting of (A) distilled water (HPLC grade) and (B) 20% v/v acetonitrile (HPLC grade). The gradient was set to vary linearly from 1% B to 99% B over a period of 18 min and remained at this level for 11 min. The gradient was then reduced to a value of 1% B over the next 3 min and then allowed to equilibrate at this level for a period of 8 min prior to the loading of the next sample.

The identities of the major peaks obtained were confirmed by comparison with those from purified glucosinolates kindly supplied by the Food Research Institute, Norwich, and response factors were determined by acid hydrolysis and subsequent glucose determination of appropriate fractions collected after separation on the HPLC as outlined by McGregor (1985).

## 3 RESULTS

### 3.1 Intracultivar variation in individual glucosinolate content

The leaves and stems from ten single plants selected at random from within a glasshouse-grown trial of the cultivar Hobson were individually analysed for glucosinolates by reversed phase HPLC. The results of this preliminary experiment (Table 1) indicated that, as has been previously shown for a number of field-

**TABLE 1**
The Concentration of the Major Glucosinolates Found in the Leaf and Stems of Individual Plants Selected from the Rape Cultivar Hobson

| *Glucosinolate* $mM\ kg^{-1}$ *dry matter* | *Leaf* | | | *Stem* | | |
|---|---|---|---|---|---|---|
| | *Range* | *Mean* | *SE* | *Range* | *Mean* | *SE* |
| 2-Hydroxy-but-3-enyl (progoitrin) | 4·0–8·1 | 5·6 | 1·44 | 15·8–22·8 | 20·4 | 2·29 |
| But-3-enyl (gluconapin) | 1·2–2·2 | 1·7 | 0·34 | 2·7–3·9 | 3·3 | 0·39 |
| 2-Hydroxy-pent-4-enyl (gluconapoleiferin) | 0·6–1·6 | 1·0 | 0·33 | 3·1–4.9 | 3·9 | 0·60 |
| Pent-4-enyl (glucobrassicanapin) | 0·6–2·5 | 1·5 | 0·64 | 2·7–3·9 | 3·3 | 0·36 |
| 3-Indole methyl (glucobrassicin) | 0·1–0·7 | 0·3 | 0·23 | 0·2–0·5 | 0·3 | 0·11 |
| 2-Phenyl ethyl (gluconasturtin) | 0·3–0·9 | 0·5 | 0·20 | 3·2–4.3 | 3·6 | 0·42 |

grown rape cultivars including Canard, Early Giant, Emerald, Samo, Winfred and Lair (Bradshaw *et al* 1984), the glucosinolate content of the stem was significantly higher than that of the leaf, with the latter having on average one-third of that found in the stem. For both leaf and stem, 2-hydroxy-but-3-enyl glucosinolate (progoitrin) amounted on average to over 50% of the total glucosinolates recovered and the other alkenyl glucosinolates to 40% and 30% of the total glucosinolates found in the leaves and stems, respectively. The aromatic glucosinolates (2-phenyl ethyl and 3-indole methyl) were present at comparatively low levels in both leaves and stems compared with progoitrin, suggesting that their nutritional significance is likely to be of only minor importance. The low levels of thiocyanate ion releasing compounds found in rape compared with kale are also reflected in the values for 3-indole methyl glucosinolate, which on average were one-tenth of those reported for a wide range of kale cultivars.

Considerable plant-to-plant variation was observed (Table 1) with respect to the levels of all the major glucosinolates present in both leaves and stems. This variation was most noticeable among the leaf samples, particularly for progoitrin which varied from 4·0 to over 8·0 mM $kg^{-1}$ dry matter. This variation, which could reflect minor differences in growing conditions, light availability or physiological state of growth, clearly suggests that no true estimate of glucosinolate content of a crop can be based on the analysis of a single plant, and consequently, for the time course study reported below, all analyses were carried out on material bulked from at least five single plants.

### 3.2 Variation in total aliphatic and total aromatic glucosinolate concentrations

The total aliphatic and total aromatic glucosinolate concentrations were determined in leaf and stem samples. Total aliphatic glucosinolate concentration was determined by summing the individual values for but-3-enyl, 2-hydroxy-but-3-enyl, pent-4-enyl and 2-hydroxy-pent-4-enyl glucosinolates, and the total aromatic glucosinolate content represented the summed values for phenyl ethyl, 3-indole methyl and 1-methoxy-3-indole methyl glucosinolates.

The results (Tables 2 and 3) indicated that, as expected, the levels of both groups of glucosinolates were lower in the leaves than the stems for all three rapes at all harvest dates. The leaf content of aliphatic glucosinolates was always greater than that of the aromatic glucosinolates, the predominance of the former tending to increase as plants of all three rapes matured. A generally similar trend was also observed for the stem samples, although in 84411 the aromatic glucosinolates represented the major proportion of the total glucosinolates on the first harvest date but thereafter the levels of aliphatic glucosinolates dominated and at week 16 accounted for over 80% of the total glucosinolates.

The variations in the concentration of leaf aliphatic and leaf aromatic glucosinolates with harvest date was broadly similar for all three rapes studied. Leaf aliphatic glucosinolates increased rapidly in concentration over the first four weeks (9–13) of the study then declined and again rose rapidly over the period week 16 to week 21, with the levels increasing from an initial value of 1·2 to 16·7 mM $kg^{-1}$ dry matter in RS5. Similar but slightly less dramatic changes were also observed in Hobson and 84411. In the final week of the study the concentrations

**TABLE 2**

Variation in the Total Aliphatic Glucosinolate Concentration (mM $kg^{-1}$ Dry Matter) of Leaves and Stems from Three Forage Rapes at Different Harvest Dates

| *Weeks from sowing* | *Leaves* | | | *Stems* | | |
|---|---|---|---|---|---|---|
| | *Hobson* | *RS5* | *84411* | *Hobson* | *RS5* | *84411* |
| 9 | 0·6 | 1·2 | 1·1 | 2·9 | 4·1 | 3·3 |
| 10 | 1·1 | 3·9 | 1·0 | 4·8 | 18·3 | 8·6 |
| 11 | 2·3 | 4·2 | 4·6 | 7·9 | 16·4 | 13·7 |
| 12 | 1·3 | 4·4 | 1·7 | 15·2 | 31·1 | 16·1 |
| 13 | 3·6 | 8·5 | 5·3 | 36·1 | 39·6 | 30·6 |
| 14 | 1·9 | 4·2 | 1·5 | 24·5 | 38·4 | 31·9 |
| 15 | 2·0 | 3·5 | 2·4 | 36·0 | 56·8 | 27·2 |
| 16 | 1·7 | 5·1 | 5·1 | 33·7 | 34·2 | 39·1 |
| 17 | 5·3 | 4·8 | 4·1 | 26·2 | 35·4 | 27·0 |
| 18 | 12·3 | 7·8 | 5·6 | 30·6 | 32·3 | 22·0 |
| 19 | 9·8 | 16·3 | 7·4 | 34·3 | 39·5 | 20·2 |
| 20 | 11·9 | 10·7 | 7·5 | 35·6 | 36·1 | 28·0 |
| 21 | 12·2 | 16·7 | 5·9 | 35·7 | 35·2 | 24·7 |
| 22 | 5·8 | 5·4 | 4·0 | 37·7 | 32·1 | 11·5 |
| LSD ($P<0·05$) | | 2·44 | | 3·48 | | |

**TABLE 3**

Variation in the Summed Aromatic Glucosinolate Concentration (mM $kg^{-1}$ dry matter) of Leaves and Stems from Three Forage Rapes at Different Harvest Dates

| *Weeks from sowing* | *Leaves* | | | *Stems* | | |
|---|---|---|---|---|---|---|
| | *Hobson* | *RS5* | *84411* | *Hobson* | *RS5* | *84411* |
| 9 | 0·3 | 0·2 | — | 2·1 | 2·2 | 5·7 |
| 10 | — | 0·2 | 0·3 | 1·2 | 3·0 | 6·7 |
| 11 | — | 0·1 | 1·1 | 1·3 | 2·0 | 6·0 |
| 12 | — | 0·1 | 0·5 | 1·8 | 2·3 | 7·1 |
| 13 | — | 0·3 | 1·2 | 4·8 | 2·7 | 9·4 |
| 14 | — | — | — | 4·0 | 2·3 | 8·7 |
| 15 | — | — | — | 6·8 | 4·4 | 8·2 |
| 16 | — | 0·1 | 0·5 | 6·9 | 2·9 | 7·8 |
| 17 | 0·2 | 0·2 | 0·3 | 2·8 | 1·5 | 3·6 |
| 18 | 0·2 | 0·3 | 0·4 | 3·8 | 2·5 | 2·4 |
| 19 | 1·1 | 1·0 | 0·5 | 3·6 | 1·2 | 3·3 |
| 20 | 0·7 | 0·6 | 1·8 | 3·9 | 1·5 | 4·0 |
| 21 | 1·1 | 0·9 | — | 4·9 | 3·5 | 3·8 |
| 22 | — | 0·1 | 0·1 | 4·0 | 1·6 | 2·0 |
| LSD ($P<0·05$) | | 0·35 | | | 0·43 | |

—, Not detected by HPLC.

of aliphatic glucosinolates in all three rapes again fell to levels similar to those found for week 16. Broadly similar trends were observed for the leaf aromatic concentrations with the levels in both RS5 and 84411 increasing up to week 13, declining and then rapidly increasing from week 16 to week 21. Hobson deviated slightly from this overall pattern to the extent that no increase in glucosinolate content was observed during the initial four weeks (9–13) of the trial. As with the leaf aliphatic glucosinolates, a rapid reduction in leaf aromatic concentration was observed for all three rapes during the last week of the study.

The aliphatic glucosinolate concentration of the stems in all three rapes increased steadily from the first week of the trial to reach a maximum around week 15 or week 16. During this 6- to 7-week period the values increased almost sevenfold, from less than 5 to over 35 mM $kg^{-1}$ dry matter. The highest value of 56·8 mM $kg^{-1}$ was found for RS5, which for the first seven weeks (weeks 9–15) of the trial had consistently higher levels of aliphatic glucosinolates than either Hobson or 84411. After this period the concentration of aliphatic glucosinolate decreased slightly in all three. The decline was most pronounced in Hobson, which in the final six weeks (weeks 16–22) fell from a peak of 39·1 to 11·5 mM $kg^{-1}$ dry matter.

The aromatic glucosinolate concentrations in the stems produced a broadly similar profile with respect to harvest date to that shown by the stem aliphatic glucosinolates. However, the rate of increase was considerably less, with the maximum value of 9·4 mM $kg^{-1}$ dry matter being recorded for 84411 which for the first nine weeks (weeks 9–17) of the trial had significantly higher concentrations of aromatic glucosinolates than either RS5 or Hobson. In the final five weeks the concentration of aromatic glucosinolates declined in all three rapes, this being most pronounced for 84411, which decreased to a final value of 2·0 mM $kg^{-1}$ dry matter at week 22.

### 3.3 Variations in individual aliphatic glucosinolate concentrations

Although the constituent aliphatic glucosinolates present in forage rape are structurally closely related, their effects *in vivo* may differ markedly and in particular the 2-hydroxy analogues of but-3-enyl and pent-4-enyl glucosinolates have been linked with the development of diet-irreversible goitre in various animal species (Fenwick *et al* 1983). Consequently the proportion of each individual glucosinolate contributing to the total glucosinolate concentration may significantly alter the nutritive value and potential toxicity of the crop at various growth stages.

An examination of the constituent aliphatic glucosinolates present in the leaves (Fig 1) revealed major differences between the three rapes studied. RS5 contained only but-3-enyl and 2-hydroxy-but-3-enyl glucosinolates, 84411 contained in addition small quantities of pent-4-enyl glucosinolate, and Hobson contained not only those three glucosinolates but also, at harvest dates where the total aliphatic concentration exceeded 2·2 mM $kg^{-1}$ dry matter, varying quantities of 2-hydroxy-pent-4-enyl glucosinolate. For all three rapes, statistically significant correlations were found between the total aliphatic glucosinolate concentrations and 2-hydroxy-but-3-enyl glucosinolate concentrations. However, considerable

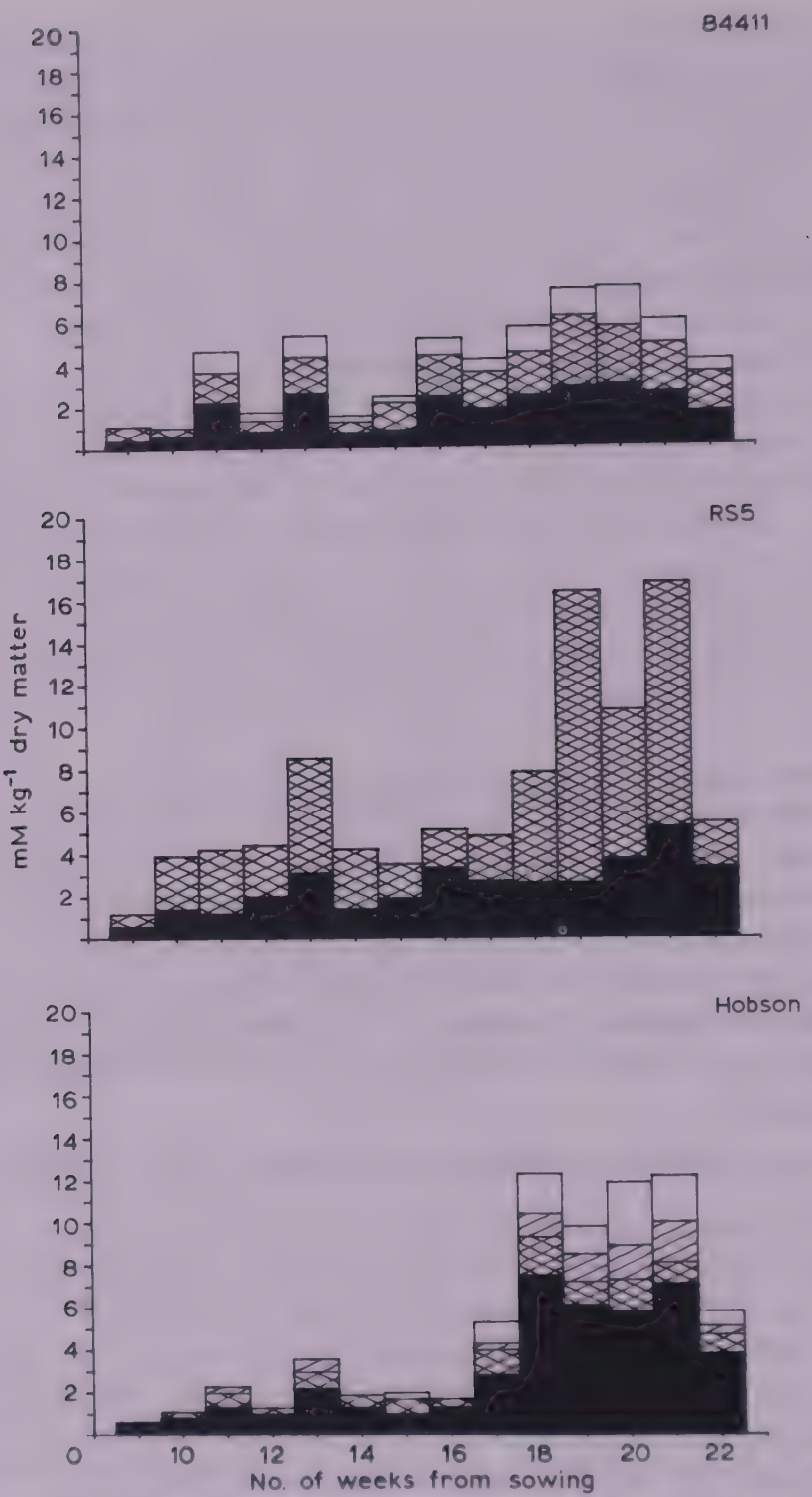


**Fig 1.** Constituent aliphatic glucosinolates present in the leaves of three forage rapes. ■ 2 Hydroxy-but-3-enyl glucosinolate; ▨ but-3-enyl glucosinolate; ◩ 2 hydroxy pent-4-enyl glucosinolate; □ pent-4-enyl glucosinolate.

variation in the proportions of 2-hydroxy-but-3-enyl glucosinolate were noted both between harvest dates and between rapes, with the mean percentage values ranging from 65·0% ±12·99 for Hobson to 43·8% ±6·43 and 41·5% ±13·51 for 84411 and RS5, respectively. The increase in total aliphatic glucosinolate concentration observed during weeks 18 to 21 was seen to be largely due to the but-3-enyl glucosinolate concentration, which increased by over eight units, whereas over

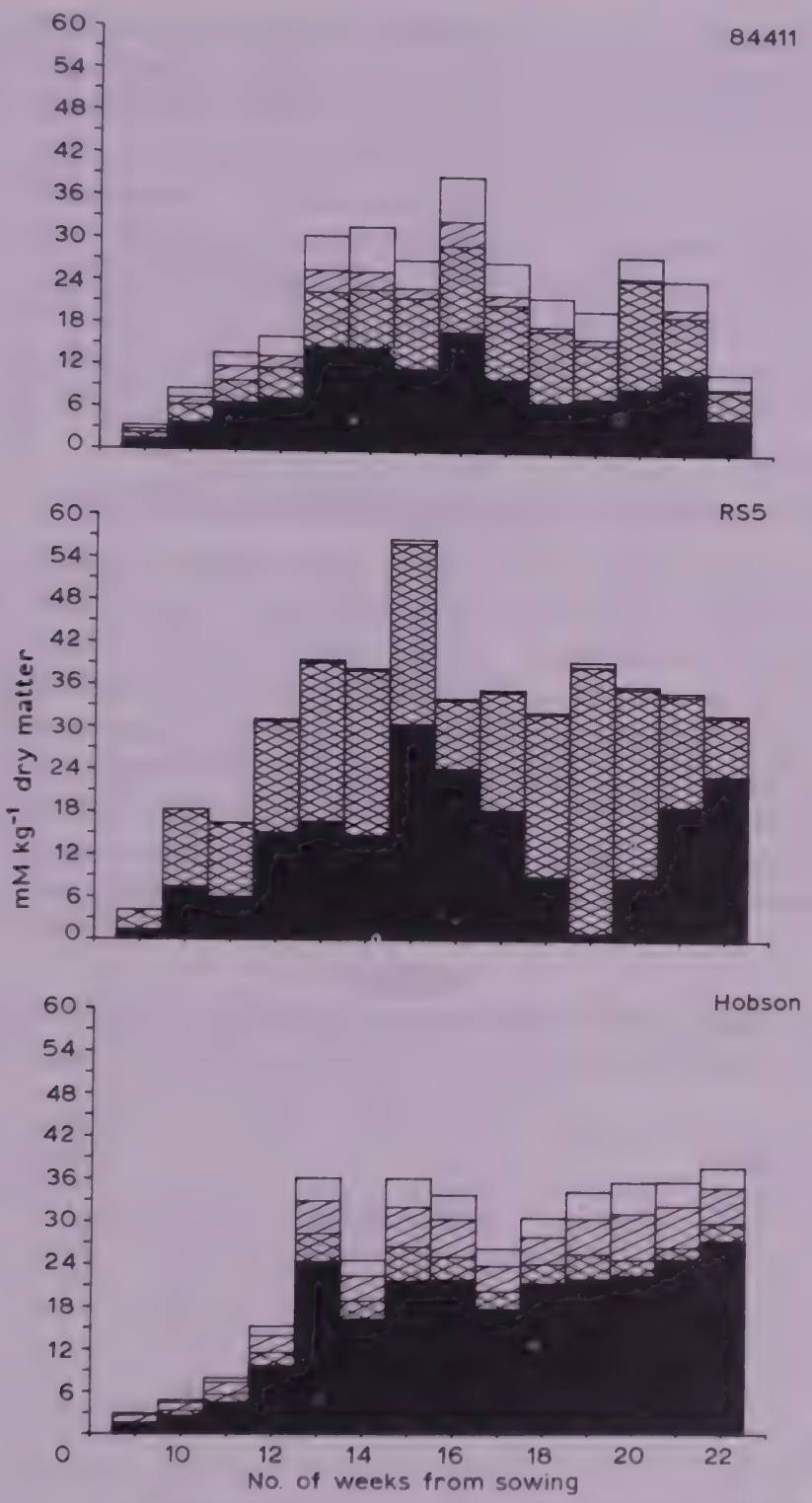


**Fig 2.** Constituent aliphatic glucosinolates present in the stems of three forage rapes. ■ 2 Hydroxy-but-3-enyl glucosinolate; ▨ but-3-enyl glucosinolate; ▤ 2 hydroxy pent-4-enyl glucosinolate; □ pent-4-enyl glucosinolate.

the same period the 2 hydroxy-but-3-enyl glucosinolate concentration increased from 2·6 to 5·2 mM $kg^{-1}$ dry matter and at week 19 accounted for only 16% of the total aliphatic glucosinolate concentration as compared with over 60% at weeks 16 and 22.

The three rapes also showed differences in aliphatic glucosinolate content in stems (Fig. 2). Hobson and 84411 contained all four aliphatic glucosinolates on all

harvest dates whereas RS5 was completely devoid of any 2-hydroxy-pent-4-enyl and only contained trace quantities of pent-4-enyl glucosinolates on harvest dates after week 11. For all three rapes, but-3-enyl and its 2-hydroxy analogue dominated, with the latter accounting for on average 62·9%±7·32, 42·6%±17·76 and 42·5%±4·94 of the total aliphatic glucosinolates in Hobson, RS5 and 84411, respectively. The intraharvest variation was greatest for RS5, where, at week 19, the 2-hydroxy-but-3-enyl glucosinolate concentration had fallen from a peak of 30·5 at week 15 to 1·0 mM $kg^{-1}$ dry matter and accounted for 2·5% of the total aliphatic glucosinolate concentration as compared with over 70% at weeks 16 and 22, a broadly similar response to that observed in the leaf samples taken from RS5 over the same growth period.

### 3.4 Variation in individual aromatic glucosinolate concentrations

The aromatic glucosinolates were present in the leaves of all three rapes only at very low concentrations. As can be seen in Table 4, 1-methoxy-3-indole methyl glucosinolate was present only at the later harvest dates for RS5 and was completely absent or present in undetectable quantities in the other two rapes. Similarly 2-phenyl ethyl glucosinolate was present in the leaves of all three rapes only in weeks 19 and 20, although trace amounts (Table 5) were detected at earlier harvest dates for 84411. The concentration of 3-indole methyl glucosinolate (Table 6) was also low in all three rapes but was detected more regularly and at higher concentrations than the other two aromatic glucosinolates.

**TABLE 4**

Variation in the 1-Methoxy-3-Indole Methyl Glucosinolate Concentration (mM $kg^{-1}$ Dry Matter) of Leaves and Stems from Three Forage Rapes at Different Harvest Dates

| *Weeks from sowing* | *Leaves* | | | *Stems* | | |
|---|---|---|---|---|---|---|
| | *Hobson* | *RS5* | *84411* | *Hobson* | *RS5* | *84411* |
| 9 | — | — | — | 0·3 | 0·5 | 0·9 |
| 10 | — | — | — | 0·1 | 0·5 | 1·1 |
| 11 | — | — | — | — | 0·3 | 1·0 |
| 12 | — | — | — | — | 0·4 | 1·2 |
| 13 | — | — | — | — | 0·5 | 1·5 |
| 14 | — | — | — | 0·1 | 0·2 | 1·2 |
| 15 | — | — | — | 0·2 | 0·7 | 1·2 |
| 16 | — | — | — | 0·2 | 0·7 | 1·1 |
| 17 | — | — | — | 0·3 | 0·4 | 0·2 |
| 18 | — | — | — | 0·3 | 0·3 | 0·4 |
| 19 | — | 0·1 | — | 0·6 | 0·1 | 0·5 |
| 20 | — | 0·1 | — | 0·6 | 0·2 | 1·1 |
| 21 | — | 0·1 | — | 0·6 | 1·2 | 0·5 |
| 22 | — | — | — | 0·9 | 0·2 | 0·5 |
| LSD ($P<0.05$) | | — | | | 0·11 | |

—, Not detected by HPLC.

**TABLE 5**

Variation in the 2-Phenyl Ethyl Glucosinolate Concentration (mM $kg^{-1}$ Dry Matter) of Leaves and Stems of Three Forage Rapes at Different Harvest Dates

| *Weeks from sowing* | *Leaves* | | | *Stems* | | |
|---|---|---|---|---|---|---|
| | *Hobson* | *RS5* | *84411* | *Hobson* | *RS5* | *84411* |
| 9 | — | — | — | 1·3 | 1·0 | 2·8 |
| 10 | — | — | — | 0·9 | 1·8 | 3·9 |
| 11 | — | — | 0·4 | 1·1 | 1·3 | 3·5 |
| 12 | — | — | 0·2 | 1·6 | 1·4 | 4·4 |
| 13 | — | — | 0·7 | 4·4 | 1·8 | 5·8 |
| 14 | — | — | — | 3·7 | 1·7 | 6·3 |
| 15 | — | — | — | 6·4 | 3·1 | 5·9 |
| 16 | — | — | 0·3 | 6·3 | 1·7 | 5·5 |
| 17 | — | — | — | 1·8 | 0·8 | 2·9 |
| 18 | — | — | 0·3 | 3·0 | 2·0 | 1·8 |
| 19 | 0·4 | 0·2 | 0·3 | 1·8 | 0·9 | 2·4 |
| 20 | 0·3 | 0·2 | 0·6 | 2·3 | 1·2 | 2·1 |
| 21 | 0·5 | 0·3 | — | 3·5 | 2·0 | 2·8 |
| 22 | — | — | — | 1·8 | 1·1 | 1·1 |
| LSD ($P<0·05$) | | 0·19 | | | 0·26 | |

—, Not detected by HPLC.

**TABLE 6**

Variation in the 3-Indole Methyl Glucosinolate Concentration (mM $kg^{-1}$ Dry Matter) of Leaves and Stems of Three Forage Rapes at Different Harvest Dates

| *Weeks from sowing* | *Leaves* | | | *Stems* | | |
|---|---|---|---|---|---|---|
| | *Hobson* | *RS5* | *84411* | *Hobson* | *RS5* | *84411* |
| 9 | 0·3 | 0·2 | — | 0·5 | 0·7 | 2·0 |
| 10 | — | 0·2 | 0·3 | 0·2 | 0·7 | 1·7 |
| 11 | — | 0·1 | 0·7 | 0·2 | 0·4 | 1·5 |
| 12 | — | 0·1 | 0·3 | 0·2 | 0·5 | 1·5 |
| 13 | — | 0·3 | 0·5 | 0·4 | 0·4 | 2·1 |
| 14 | — | — | — | 0·2 | 0·4 | 1·2 |
| 15 | — | — | — | 0·2 | 0·6 | 1·1 |
| 16 | — | 0·1 | 0·2 | 0·4 | 0·5 | 1·2 |
| 17 | 0·2 | 0·2 | — | 0·7 | 0·3 | 0·5 |
| 18 | 0·2 | 0·3 | 0·1 | 0·5 | 0·2 | 0·2 |
| 19 | 0·7 | 0·7 | 0·2 | 1·2 | 0·2 | 0·4 |
| 20 | 0·4 | 0·3 | 1·2 | 1·0 | 0·1 | 0·8 |
| 21 | 0·6 | 0·5 | — | 0·8 | 0·3 | 0·5 |
| 22 | — | 0·1 | 0·1 | 1·3 | 0·3 | 0·4 |
| LSD ($P<0·05$) | | 0·08 | | | 0·14 | |

—, Not detected by HPLC.

In contrast, significantly higher concentrations of all three aromatic glucosinolates were found in the stem samples (Tables 4, 5 and 6), particularly with respect to 2-phenyl ethyl glucosinolate, which accounted for on average, over all harvest dates, 74·9%±16·02, 65·4%±9·77 and 65·4%±9·4 of the total aromatic glucosinolates present in Hobson, RS5 and 84411, respectively. The concentration of this glucosinolate varied significantly both between rapes and between harvest dates but the highest concentrations were generally associated with Hobson and 84411, both of which peaked at values exceeding 6·0 mM $kg^{-1}$ dry matter during the period weeks 12–16, as compared with a maximum value of 3·1 mM $kg^{-1}$ dry matter recorded for RS5 during the same growth period. The highest concentrations of both indole-containing glucosinolates, particularly during the earlier phases of growth (weeks 9–17) were consistently associated with 84411, whereas for the other two rapes the concentrations of both indole glucosinolates only just exceeded 1·0 mM $kg^{-1}$ dry matter on two harvest dates.

## 4 DISCUSSION

The low concentrations of aromatic glucosinolates, and in particular those of the indole-containing glucosinolates, clearly suggest that the nutritional significance of thiocyanate ion-induced, diet-reversible goitre is unlikely to be great in relation to the feeding of forage rape. The concentration of both 3-indole methyl and 2-hydroxy-3-indole methyl glucosinolates remained comparatively low in both stems and leaves, indicating that, although the ratio of stem to leaf increased with maturity, the levels present in the whole plant remained considerably less than those reported for various kale cultivars. Consequently even if the crop was grazed at high stocking rates where the consumption of stem may be significantly increased, the intake of thiocyanate ion releasing compounds would be expected to remain comparatively low.

Significant differences were, however, observed between the three rapes examined with respect to the aliphatic glucosinolate content. The results for Hobson showed that it was a typical forage rape cultivar with the same major glucosinolates as found in six other forage rape cultivars by Bradshaw *et al* (1984). The leaves of RS5 contained only but-3-enyl and 2-hydroxy-but-3-enyl glucosinolates, whereas the stem contained in addition trace quantities of pent-4-enyl glucosinolate. The almost complete absence or at least very significant reduction in pent-4-enyl glucosinolate and the complete absence of its 2-hydroxy analogue in this line possibly indicates that if, as previously outlined (Gland *et al* 1981; see Fig 3), aliphatic glucosinolates are in the same synthetic pathway, the enzymic elongation of the precursor of but-3-enyl to the precursor of pent-4-enyl is severely inhibited in RS5 compared with either Hobson or 84411. Additionally RS5 also displayed a marked reduction in both the concentration and proportion of 2-hydroxy-but-3-enyl during weeks 16–19, which was particularly evident in the stem and did not coincide with any sudden change in the plant growth rate. This reduction was accompanied by a corresponding increase in the concentration of but-3-enyl glucosinolate, the precursor of the 2-hydroxy analogue, suggesting that

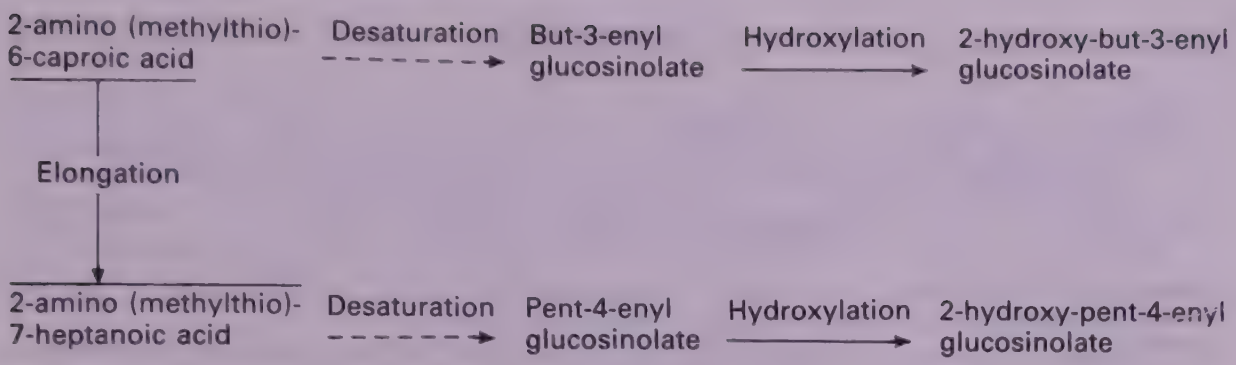


**Fig 3.** The biosynthetic interrelationship between the major constituent alkyl glucosinolates present in forage rape (adapted from Gland *et al* 1981).

for some reason the conversion of the former to the latter temporarily ceased during this period. RS5 has a similar pedigree to another forage rape breeding line, AR/5/7/10/1, which was shown previously (Bradshaw *et al* 1983) also to have a higher concentration of 3-butenyl than 2-hydroxy-3-butenyl glucosinolate. This may therefore be a characteristic of this type of resynthesised *B napus*. It is clear that further physiological and biochemical studies are required to determine why such a process was suddenly terminated and whether it merely reflects the environmental conditions appertaining at the time (although these would have been identical for all three rapes) or whether there was a more fundamental underlying mechanism.

The observed variation in both the proportion and concentration of individual glucosinolates indicates that individual glucosinolate concentration cannot be accurately determined from predictions based on the determination of total glucosinolate content by the rapid screening methods currently available. The variations in concentration of the individual glucosinolates with harvest date also suggest that extreme care in harvesting plants at similar stages of growth is required if any meaningful comparison of cultivars and breeding selections is to be made. Indeed before any real progress in selecting for reduced 2-hydroxy-but-3-enyl glucosinolate concentration can be claimed, a time-course study may be necessary. Clearly further work under field conditions is required to determine more precisely the effects of environment on individual glucosinolate production so that a more accurate estimation of the potential toxicity of forage rape crops may be made, as well as to evaluate the possibility of the utilisation of various agronomic practices to control and limit the goitrogenic potential of the crop.

Work currently in progress is intended to demonstrate the precise importance of 2-hydroxy-but-3-enyl glucosinolate (progoitrin) in the nutrition of sheep. However, it is clear that progress has been made in reducing the level of a potential anti-metabolite. This has required the resynthesis of *B napus*, a process which not only has more technical difficulties (McNaughton and Ross 1978) but is also more costly than conventional plant breeding. However, screening of the parental species *B campestris* and *B oleracea* has demonstrated the existence of cultivars in which progoitrin is present at very low levels, if at all. There are therefore good prospects for producing a zero-progoitrin form of *B napus*.

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# Phenolic Compounds in Coffee Pulp: Quantitative Determination by HPLC

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*ABSTRACT*

*The content of phenolic compounds tentatively identified by HPLC in fresh coffee pulp gives an average composition in the 12 cultivars studied as follows: chlorogenic acid (5-caffeoylquinic acid), 42·2%; epicatechin, 21·6%; isochlorogenic acid I, 5·7%; isochlorogenic acid II, 19·3%; isochlorogenic acid III, 4·4%; catechin, 2·2%; rutin, 2·1%; protocatechuic acid, 1·6%; and ferulic acid, 1·0%. When the percentages of chlorogenic and isochlorogenic acids are added to the corresponding one of epicatechin for each cultivar, it is found that they make up between 92·0% and 98·4% of the total of identified phenolic compounds. Qualitative or quantitative differences were not detected between cultivars of coffee plants resistant and susceptible to coffee leaf rust.*

*Key words*: Coffee, pulp, phenols, flavonoids, HPLC.

## 1 INTRODUCTION

Coffee pulp has been extensively studied from an animal nutrition point of view (Braham and Bressani 1979). However, it is not utilised widely for animal feeding because of the presence of unknown toxic substances. One of the conjectures about the nature of these toxic substances is that they could be phenolic compounds, which are very abundant in coffee beans (Bressani 1979). In spite of this, information about phenolic compounds in coffee pulp continues to be both



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insufficient and inconsistent. Thus, there is a need for further studies of the chemical composition of coffee pulp and especially of its phenolic compounds.

High-performance liquid chromatography (HPLC) has been displacing progressively other chromatographic techniques for the separation, identification and quantification of phenolic compounds (Wulf and Nagel 1976; Hartley and Buchan 1979; Nagels *et al* 1980; Villeneuve *et al* 1982; Mollerup-Anderson and Batsberg-Pedersen 1983; Vande-Casteele *et al* 1983) because of its sensitivity, resolution power, non-destructive nature and relative speed (Wulf and Nagel 1976; Schwartz and von Elber 1982). In some cases it has been used for the separation of complex mixtures of these compounds present in plant material extracts (Hardin and Stutte 1980; Moller and Herrmann 1982; Winter and Herrmann 1984). In the genus *Coffea* HPLC has been utilised to study chlorogenic acids in coffee beans (Van der Steegen and Van Duijn 1979; Morishita *et al* 1984; Iwahashi *et al* 1985). In the case of coffee pulp, where phenolic compounds are so varied and some of them exiguous (Ramirez-Martinez in press), identification and quantification could be facilitated by the use of HPLC.

For the analysis of phenolic compounds with this technique, many systems have been described that utilise octadecyl groups bound to silica (ODS columns) as stationary phase and either a fixed (isocratic elution) or changing (gradient elution) mixture of water and alcohol (usually methanol) as mobile phase (Burtscher *et al* 1982; Brandl and Herrmann 1983). These systems correspond to the so-called reversed phase column chromatography, where the mobile phase is more polar than the stationary phase.

As a continuation of the studies aimed to determine the nature and content of phenolic compounds in coffee pulp, this paper presents the results of the analytical separation and quantitative determination by HPLC of some phenolic compounds present in fresh coffee pulp of 12 cultivars grown either commercially or experimentally in the Andes region of Venezuela.

## 2 EXPERIMENTAL

### 2.1 Materials

Ripe fruits of *Coffea arabica* L were obtained from the Estación Experimental de Bramón, Estado Táchira, Venezuela. Eight cultivars susceptible to coffee leaf rust (Yellow and Red Bourbon, Yellow and Red Caturra, Yellow Catuai, Red Typica, Mundo Novo 385 and Semperflorens) and four cultivars resistant to some strains of this disease (BA-2, Geisha B, KP-263 and KP-423) were chosen for these studies.

One day after harvesting, the ripe fruits were depulped by hand and 100 g of pulp was immediately extracted with 200 ml of 80% methanol containing 0·005 M cysteine. The residue was extracted similarly three more times. Methanol was removed from the extract with a Buchii rotavapor and the aqueous suspension was first extracted with ethyl acetate (fraction A) and then with a mixture of methanol and ethyl acetate (fraction B) as described elsewhere (Ramirez-Martinez in press).

### 2.2 Methods of analysis

Analytical separation and quantitative determination of phenolic compounds were performed in Perkin-Elmer HPLC equipment composed of a liquid chromatograph Series 2, a UV light detector, model LC-75, a Rheodyne 7105 injection valve and a laboratory computing integrator model LCI-100. A 25×0·26 cm ID reversed phase liquid chromatography column (HC ODS/SIL-X, Perkin-Elmer) enclosed in a glass jacket with water circulating at 25°C (Bowermaster and McNair 1984) was used as stationary phase. A mixture of two solvents was utilised as mobile phase: solvent A (absolute methanol, HPLC grade) and solvent B (2% acetic acid in aqueous solution). The water of solvent B was distilled directly to a Barnstead demineraliser (Arthur Thomas Co, Philadelphia, PA, USA) and afterwards filtered through a Norganic cartridge (Millipore Corp, Bedford, MA, USA) and a 0·45 $\mu$m membrane Millipore filter (Type HATF). The acetic acid of solvent B and each sample to be analysed was filtered through a 0·5-$\mu$m Millipore filter (Type FHLP).

The HPLC analytical separation was achieved by combining linear gradient elution with isocratic elution: 0–5 min, 12 to 15% of A in B (linear gradient); 5–15 min, 15–30% of A in B (linear gradient); 15–19 min, 30% of A in B (isocratic); 19–27 min, 30–50% of A in B (linear gradient). At a flow of 0·5 ml $min^{-1}$ the effective concentration is reached after 9 min, and therefore the first 9-min period actually corresponds to an isocratic elution of 12% of A in B. Thus, total elution time was 36 min. The UV light detector was operated at a wavelength of 280 nm and with a sensitivity of 0·16 AUFS. Size of the injected sample was constant at 5 $\mu$l.

The method of external standards was used for the quantitative determination of the tentatively identified phenolic compounds. Values for the retention time and response factor for each external standard substance were obtained by calibration chromatographic runs. The following external standards were used: caffeine, prepared in the laboratory from tea leaves; catechin, chlorogenic acid, caffeic acid, epicatechin, *p*-coumaric acid and ferulic acid purchased from Sigma (St Louis, Mo, USA); and protocatechuic acid, rutin and isochlorogenic acid kindly donated by Dr Eckbert Schulz-Schomburgk (Facultad de Agronomía, UCV, Maracay, Venezuela).

## 3 RESULTS

Table 1 presents for each one of the external standards used the corresponding peak number, retention time and response factor obtained from calibration runs with the HPLC equipment. The numbers of the peaks were assigned according to the order of appearance in the chromatographic run. The response factor is the ratio between the concentration of the external standard and the area of its corresponding peak in the calibration chromatogram.

Figure 1 is a diagram of an actual calibration chromatogram run using external standards concentrations in such a proportion that its outline resembles that of the chromatograms of fraction A from the pulp of any of the cultivars studied. This

**TABLE 1**
Peak Number, Retention Time ($R_t$) and Response Factor ($R_f$) of Reference Compounds

| *Compound* | *Peak* | $R_t$ *(min)* | $R_f \times 10^8$ |
|---|---|---|---|
| Protocatechuic | 1 | 4·56 | 28·332 |
| Catechin | 3 | 6·14 | 65·47339 |
| Chlorogenic acid | 5 | 8·54 | 30·6578 |
| Caffeic acid | 6 | 9·90 | 11·6822 |
| Epicatechin | 9 | 13·58 | 89·1969 |
| Caffeine | 11 | 15·50 | 24·786 |
| *p*-Coumaric acid | 12 | 17·25 | 4.74815 |
| Ferulic acid | 16 | 20·67 | 9.204829 |
| Isochlorogenic acid I | 21 | 27·76 | 77·05069 |
| Isochlorogenic acid II | 22 | 28·66 | 77·05069 |
| Rutin | 24 | 29·60 | 65·1317 |
| Isochlorogenic acid III | 26 | 32·77 | 77·05069 |

was made on purpose to take into account the effect of high concentrations of known substances, such as caffeine, on the separation of the other substances.

Figure 2 shows the chromatogram corresponding to fraction A of Red Bourbon. The most conspicuous peaks (amounting to around 95% of the integrated area) were tentatively identified as follows: 1, protocatechuic acid; 3, catechin; 5,

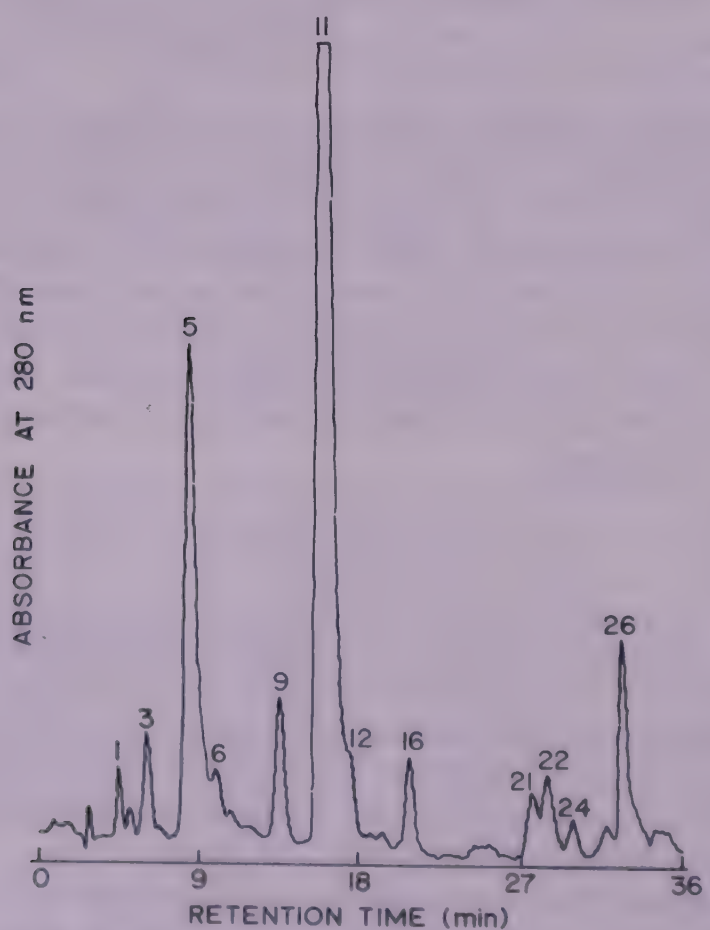


**Fig 1.** Chromatogram of caffeine and nine reference phenolic compounds (peak identification according to Table 1).

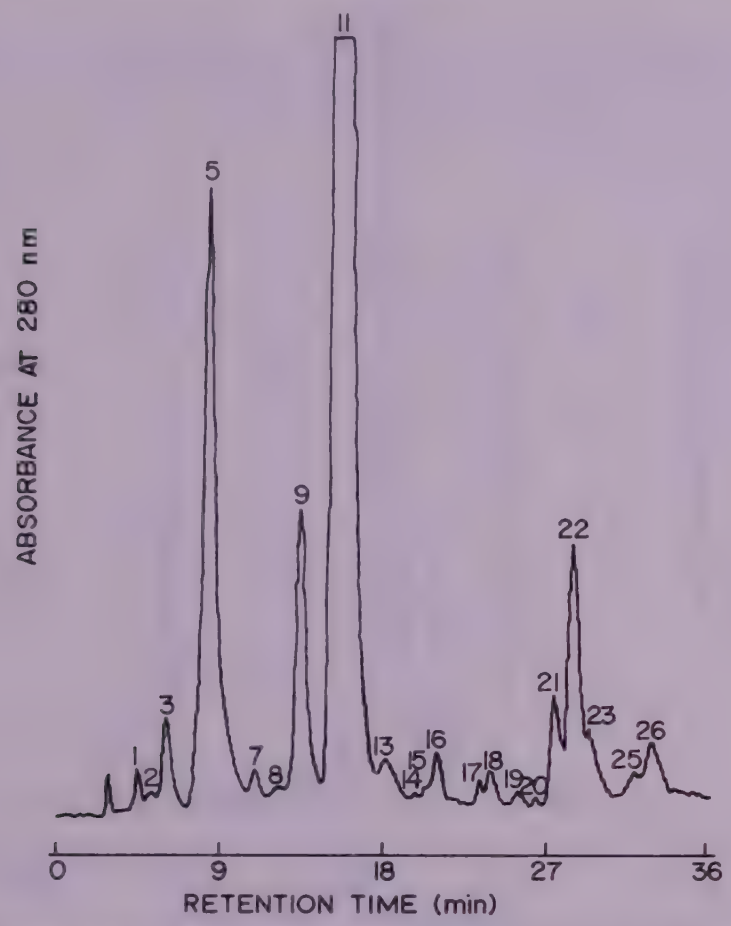


**Fig 2.** Chromatogram of the ethyl acetate extract (fraction A) from Red Bourbon coffee pulp.

chlorogenic acid (5-caffeoylquinic acid, IUPAC 1976, commonly but incorrectly designated 3-caffeoylquinic acid); 9, epicatechin; 11, caffeine; 16, ferulic acid; and 21, 22 and 26, isochlorogenic acids I, II and III, respectively. Figure 3 is a diagram of the chromatogram corresponding to fraction A of Yellow Bourbon. Peaks 5, 9, 11, 16, 21, 22 and 26 are again prominent and account for about 95% of the integrated area.

When the Fraction A chromatograms for Red and Yellow Bourbon are compared, it is noticed that they show minimal qualitative differences. Peak 3 (catechin) is relatively large in Red Bourbon, whereas in Yellow Bourbon it is relatively small and close to it appears peak 4 which is absent in Red Bourbon. Peaks 7 and 8 are relatively small in Red Bourbon, whereas in Yellow Bourbon peak 7 is absent and peak 8 is relatively large.

The chromatogram corresponding to Fraction B of Red Bourbon is presented in Fig 4 and is representative of both the red and yellow cultivars studied. Only the most prominent peaks, representing around 80% of the integrated area, were numbered and tentatively identified as follows: 1, protocatechuic acid; 5, chlorogenic acid (5-caffeoylquinic acid); 11, caffeine; 16, ferulic acid; and 24, rutin. Qualitative differences were not found among the 12 cultivars studied.

Values for the concentration of tentatively identified phenolic compounds in fresh coffee pulp of eight susceptible cultivars and four resistant cultivars to coffee leaf rust are presented in Tables 2 and 3, respectively. The values are averages from three to five analyses. The data shown indicate that there is no evident

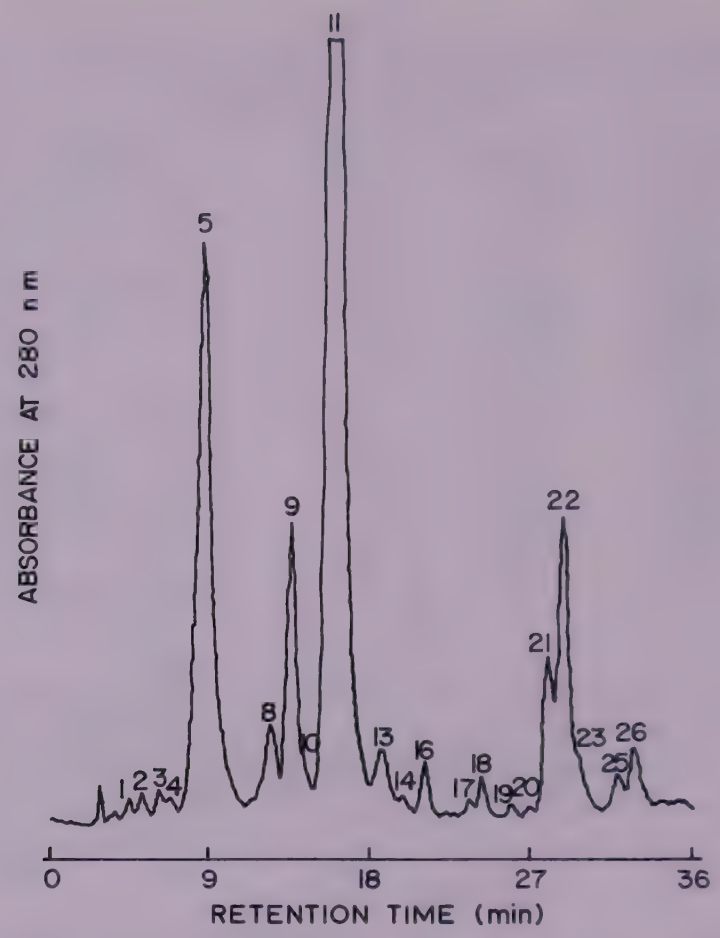


**Fig 3.** Chromatogram of the ethyl acetate extract (fraction A) from Yellow Bourbon coffee pulp.

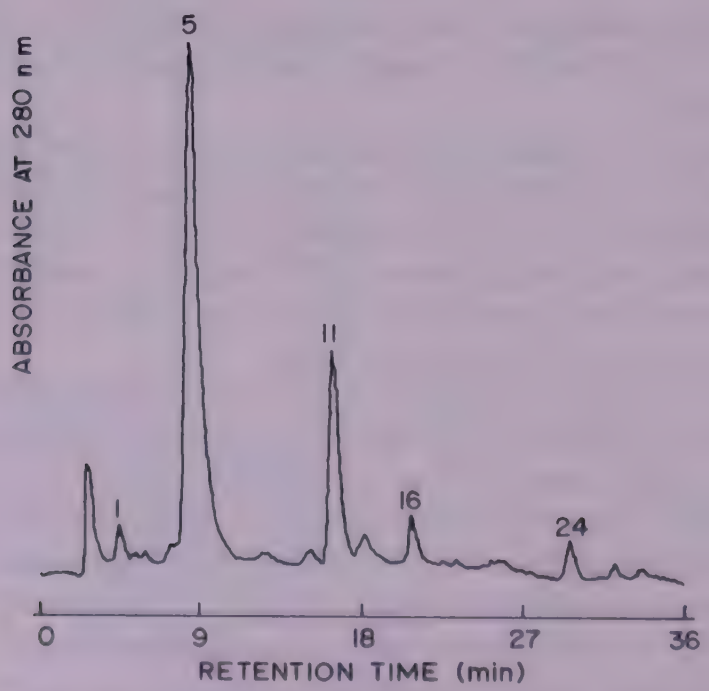


**Fig 4.** Chromatogram of the ethyl acetate plus methanol extract (fraction B) from Red Bourbon coffee pulp.

**TABLE 2**
Phenolic Compounds Concentration (g $kg^{-1}$) on a Dry Matter Basis in Fresh Coffee Pulp of Eight Cultivars Susceptible to Leaf Rust

| *Phenolic compound* | *Cultivar* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Yellow Bourbon* | *Red Bourbon* | *Yellow Catuai* | *Yellow Caturra* | *Red Caturra* | *Mundo Novo 385* | *Semper-florens* | *Red Typica* |
| Protocatechuic acid | 0·11 | 0·20 | 0·15 | 0·09 | 0·20 | 0·18 | 0·13 | 0·31 |
| Catechin | 0·07 | 0·58 | 0·06 | 0·06 | 0·31 | 0·23 | 0·18 | 0·60 |
| Chlorogenic acid | 3·86 | 7·39 | 5·88 | 2·38 | 4·64 | 6·82 | 5·12 | 8·76 |
| Epicatechin | 1·85 | 4·36 | 1·53 | 1·87 | 2·72 | 3·21 | 1·71 | 3·88 |
| Ferulic acid | 0·05 | 0·13 | 0·10 | 0·11 | 0·17 | 0·12 | 0·09 | 0·21 |
| Isochlorogenic acid I | 0·77 | 1·13 | 0·65 | 0·30 | 0·67 | 0·51 | 0·49 | 0·95 |
| Isochlorogenic acid II | 2·19 | 4·45 | 2·52 | 0·90 | 2·28 | 2·08 | 2·02 | 3·70 |
| Isochlorogenic acid III | 0·43 | 1·22 | 0·50 | 0·29 | 0·67 | 0·52 | 0·39 | 0·70 |
| Rutin | 0·12 | 0·38 | 0·23 | 0·09 | 0·23 | 0·33 | 0·21 | 0·70 |
| Total | 9·45 | 19·84 | 11·62 | 6·09 | 11·89 | 14·00 | 10·34 | 19.81 |

difference between cultivars susceptible and resistant to leaf rust with regard to the composition and content of the most abundant phenolic compounds in fresh coffee pulp. Differences in the content of each one of the identified phenolic compounds do exist among the different cultivars studied but, if this content is expressed as a fraction of the total content of identified phenolic compounds, a tendency towards an average composition is observed.

Table 4 presents the average composition values of the phenolic compounds for fractions A and B of the fresh coffee pulp of all the cultivars studied and the range for these values. Average composition for the tentatively identified phenolic compounds in fractions A and B combined is as follows: chlorogenic acid (5-caffeoylquinic acid), 42·2%; epicatechin, 21·6%; isochlorogenic acid I, 5·7%;

**TABLE 3**
Phenolic Compounds Concentration (g $kg^{-1}$) on a Dry Matter Basis in Fresh Coffee Pulp of Four Cultivars Resistant to Leaf Rust

| *Phenolic compound* | *Cultivar* | | | |
|---|---|---|---|---|
| | *BA-2* | *Geisha B* | *KP-263* | *KP-423* |
| Protocatechuic acid | 0·35 | 0·27 | 0·21 | 0·19 |
| Catechin | 0·63 | 0·18 | 0·41 | 0·23 |
| Chlorogenic acid | 5·41 | 7·40 | 3·48 | 3·52 |
| Epicatechin | 3·11 | 3·40 | 2·64 | 2·10 |
| Ferulic acid | 0·09 | 0·10 | 0·11 | 0·11 |
| Isochlorogenic acid I | 0·86 | 0·76 | 0·78 | 0·57 |
| Isochlorogenic acid II | 2·79 | 3·48 | 1·89 | 1·61 |
| Isochlorogenic acid III | 0·64 | 0·59 | 0·51 | 0·30 |
| Rutin | 0·24 | 0·30 | 0·21 | 0·29 |
| Total | 14·12 | 16·48 | 10·24 | 8·92 |

**TABLE 4**
Phenolic Compounds in Fractions A and B from Fresh Coffee Pulp: Mean % Total Phenolics (Range)

| *Phenolic compound* | *Fraction A* | *Fraction B* |
|---|---|---|
| Protocatechuic acid | 0·8 (0·3–1·4) | 3·8 (2·8–4·7) |
| Catechin | 3·0 (0·8–5.6) | — |
| Chlorogenic acid | 25·2 (17·4–35·0) | 86·5 (80·7–90·5) |
| Epicatechin | 29·8 (21·4–41·3) | — |
| Ferulic acid | 0·5 (0·2–1·8) | 2·1 (1·1–3·6) |
| Isochlorogenic acid I | 7·8 (5·2–10·8) | — |
| Isochlorogenic acid II | 26·8 (20·0–30·7) | — |
| Isochlorogenic acid III | 6·1 (5·1–7·7) | — |
| Rutin | — | 7·7 (5·0–11·5) |

isochlorogenic acid II, 19·3%; isochlorogenic acid III, 4·4%; catechin, 2·2%; rutin, 2·1%; protocatechuic acid, 1·6%; and ferulic acid, 1·0%. If the concentration values for chlorogenic and isochlorogenic acids are added to that of epicatechin in each cultivar studied, it is found that they constitute between 92·0 and 98·4% of the phenolic compounds identified in fresh coffee pulp.

## 4 DISCUSSION

Solvent choice for HPLC separation of phenolic compounds was based on cost and facility to be either obtained commercially or purified locally, as well as on their previous use elsewhere (Daigle and Conkerton 1982; Moller and Herrmann 1982; Brandl and Herrmann 1983). Conditions for the chromatographic runs were established by trial and error until all substances used as standards and the largest possible number of components of the unknown samples were satisfactorily separated. In the latter there is the possibility that some of the peaks could be made up of more than one component. In both fractions A and B from fresh coffee pulp, the majority of the peaks with large areas were those tentatively identified and comprised around 95 and 80%, respectively, of the total area produced by compounds absorbing light at 280 nm.

HPLC enabled not only the verification but also the quantification of the observations made by paper chromatography on simple phenolic compounds abundant in fresh coffee pulp (Ramirez-Martinez in press). Furthermore, it was possible to detect compounds that were not revealed by paper chromatography, such as protocatechuic and ferulic acids. On the other hand, the presence of free caffeic acid in coffee pulp, reported by Molina *et al* (1974a, b) and Lopes *et al* (1984) was not confirmed by either paper chromatography (Ramirez-Martinez in press) or HPLC. The presence of caffeic acid could have resulted from the hydrolysis of chlorogenic acids occurring when fresh coffee pulp is piled, dried or extracted under conditions that favour chemical changes. It is interesting to note that Griffin and Stonier (1975) never found caffeic acid and chlorogenic acid together in the same extract of coffee pulp.

Chlorogenic acid (5-caffeoylquinic acid), which is the most abundant simple

phenolic compound in fresh coffee pulp, varied on a dry matter basis between 0·24% for Yellow Caturra and 0·88% for Red Typica. If isochlorogenic acid, another abundant phenolic compound, is added to chlorogenic acid, then the value for total chlorogenic acids varies between 0·39% for Yellow Caturra and 1·47% for Red Bourbon. These figures are certainly well below those of 2.71% and 2·60% given by Moline *et al* (1974a, b). These higher values were found by using spectrophotometry on spots eluted from paper chromatograms.

The individual identification of the isochlorogenic acid isomers was impracticable because of the lack of the corresponding reference compounds. However, based upon studies on chlorogenic acids from coffee beans by Morishita *et al* (1984), who used a similar solvent system, it seems that isochlorogenic acid I is 3,4-dicaffeoylquinic acid, isochlorogenic acid II is 3,5-dicaffeoylquinic acid and isochlorogenic acid III is 4,5-dicaffeoylquinic acid (cf Adzet and Puigmacia 1985).

Another abundant phenolic compound in fresh coffee pulp appeared to be epicatechin, which is a well-known constituent of condensed tannins. Epicatechin content was relatively low in yellow coffee pulp compared with that in most of the red ones. Rutin, which is a diglycoside of quercetin, appeared only in fraction B of fresh coffee pulp at relatively low concentrations, except in Red Typica where it was relatively high.

Finally, the overall results indicate that simple phenolic compounds are relatively abundant in fresh coffee pulp, especially chlorogenic acids and epicatechin. It should be borne in mind that content of the different phenolic compounds present in fresh coffee pulp could vary from year to year, from day to day and even from hour to hour (El-Hamidi and Wanner 1964). Hence, the phenolic compound contents reported here are not absolute and, therefore, it would be pointless to try to establish quantitative comparisons among coffee pulps from different cultivars. Moreover, identification of each phenolic compound is provisional, and it should be validated with the aid of preparative HPLC mass spectrometry and nuclear magnetic resonance.

## ACKNOWLEDGEMENT

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# Dietary Manipulation of the Yield and Composition of Milk: Effects of Dietary Inclusions of Barley and Oats in Untreated or Formaldehyde-treated Forms on Milk Fatty Acid Composition

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*ABSTRACT*

*Eight 1st-lactation cows were given four dietary treatments in a duplicated 4×4 Latin square experiment. Diets consisted of hay and soya bean meal together with barley, formaldehyde-treated barley, oats or formaldehyde-treated oats (approximately 34:12:54 on a dry matter basis). Barley diets supplied 211 g fatty acids $d^{-1}$, oats diets supplied 537 g $d^{-1}$. The fatty acid composition (g $kg^{-1}$ total fatty acids) for barley diets was: 300 (16:0); 20 (18:0); 150 (18:1); 470 (18:2); 60 (18:3). Corresponding values for oats diets were 180, 20, 390, 380 and 30 g $kg^{-1}$.*

*Formaldehyde treatment of the cereals tended to increase milk yield and reduce milk fat content ($P<0.01$ for barley) but did not affect milk fatty acid composition. Feeding oats in replacement for barley significantly ($P<0.05$) increased milk yield and lactose yield and reduced milk fat content ($P<0.05$ for the untreated cereals) and protein contents ($P<0.01$) without significant effects on milk fat or protein yields. Oats diets led to significant ($P<0.001$) reductions in the content of 8:0–16:0 fatty acids in milk fat with associated increases ($P<0.001$) in the content of 18:0 and 18:1. Changes in milk fat content of 18:2 and 18:3 acids were small.*

*The results show the inclusion of oats in the cow's diet to be a means of reducing the saturated fatty acid content of milk fat thereby improving the nutritional value of milk and milk products and their appeal to the health-conscious consumer.*

*Key words*: Milk yield, milk fat composition, oats, barley, formaldehyde treatment.

## 1 INTRODUCTION

Concern over the suggested links between consumption of saturated fats and the development of coronary heart disease and other disorders in man has prompted recommendations that their consumption be reduced (NACNE 1983; Committee on Medical Aspects of Food Policy 1984). Low-fat milks and low-fat dairy foods are increasing in popularity, but pressure to reduce the saturated fat content of milk and dairy products is likely to continue; the high saturated fatty acid content and low polyunsaturated fatty acid content of milk fat is seen as a contributory factor to the declining consumption of butter and as a barrier to the use of milk fat in human foods generally.

Marked reductions in the saturated fatty acid content of milk fat can be achieved through the inclusion of selected sources of 'free' fats in the cow's diet (Banks *et al* 1980; Clapperton and Banks 1985). Also increases in polyunsaturated fatty acid content can be effected using dietary supplements of 'rumen-protected' polyunsaturated fats, coated to avoid the effects of microbial biohydrogenation in the rumen (Scott and Cook 1975). However, the difficulties of handling free fats in farm-mixed rations and the adverse effects of some of the fats on milk fat content (Banks *et al* 1980) militate against their widespread acceptance, and the cost and technological problems associated with the production and use of protected polyunsaturated fats has prevented their commercial exploitation.

A simple and inexpensive approach to the dietary manipulation of milk fatty acid composition is required, and with that in view we have examined the possible role of oats as a cereal inclusion in dairy cow rations. Oats commonly contain almost twice as much lipid as other cereals (principally barley and wheat) grown in the UK as feeds for dairy cows, and up to half of the fatty acids in oats may be polyunsaturated (Morrison 1977). Transfer of those fatty acids to milk either directly or via biohydrogenation in the rumen and subsequent desaturation in the mammary gland would lead to beneficial changes in milk fat composition (Christie 1981). The experiment reported involved a comparison between oats and barley with the rolled cereals provided either in untreated form or chemically treated with an acidified formaldehyde reagent. The reagent is known to cross-link cereal starch and protein, reducing both the rate and extent of their degradation in the rumen (Kassem *et al* 1987; Van Ramshorst and Thomas 1988) and it seemed possible that this cross-link matrix might confer on the cereal lipid a degree of protection against ruminal biohydrogenation by creating a physical barrier between the lipid and the rumen microorganisms.

## 2 MATERIALS AND METHODS

### 2.1 Animals

Eight 1st-lactation Friesian cows were used. The animals, which were in the 6th–10th week of lactation at the start of the experiment, had an average weight of 456 kg. They were housed in individual standings in a cattle byre and were fed and milked twice daily at 06.00 and 16.00 h. Drinking water was freely available.

### 2.2 Experimental design and treatments

The experiment was conducted according to a duplicated 4×4 Latin square design with four dietary treatments and four 3-week experimental periods. The diets consisted of hay and a concentrate mixture containing soya-bean meal with rolled barley or rolled oats included in either untreated or formaldehyde-treated forms. The proportions of hay, soya bean meal and cereal in the diets were approximately 34:12:54 on a dry matter (DM) basis. The formaldehyde treatment of the cereal was undertaken as previously described (Kassem *et al* 1987; Van Ramshorst and Thomas 1988), with a reagent application rate of 15 litres $t^{-1}$. Cows were rationed at a level to provide a calculated metabolisable energy (ME) intake of 145 MJ $d^{-1}$, and this level of feeding was held constant throughout the experiment. All diets were supplemented with a proprietary mineral mixture to meet the animals' requirements (Agricultural Research Council 1980).

### 2.3 Recording and sampling

Feed intake and milk yield were measured daily; quoted results are based on intakes for the last 14 days and milk yields for the last 7 days of each experimental period. Samples of milk were taken at the last four milkings of each period and bulked in proportion to yield to provide a sample for analysis.

### 2.4 Chemical analysis

Feedstuffs were analysed for DM by drying at 100°C to constant weight and for organic matter by ashing in a muffle furnace at 550°C overnight. The digestible organic matter in the dry matter (DOMD) of the hay was measured on the basis of lignin content by the method of Morrison (1972). Total nitrogen was determined by a Kjeldahl method (MAFF 1981). Fatty acid content and composition of feedstuffs were determined as by Steele (1980). Milk samples were analysed for total solids gravimetrically, for total protein (N×6·38) by a Kjeldahl method, for lactose polarimetrically (Grimbleby 1956) and for fatty acids by gas–liquid chromatography (Thomas and Kelly 1976). *Cis* and *trans* isomers of monoenoic fatty acids were separated by silver nitrate thin-layer chromatography (Christie and Moore 1971). Bands containing saturated and *trans* monoenoic fatty acids were scraped into micro test tubes and the methyl esters were recovered in chloroform:methanol (98:2 on a volume basis) by centrifugation. The concentration of *trans* monoenoic fatty acids was determined by comparing the result of gas chromatography of the extract with that of the unfractionated sample.

### 2.5 Statistical analysis

Results were analysed by analysis of variance for a duplicated 4×4 Latin square (Snedecor and Cochran 1980).

## 3 RESULTS

### 3.1 Diet composition and intake

The composition of the dietary ingredients is shown in Table 1. As expected, the total fatty acid content of the oats was substantially greater than that of the barley.

**TABLE 1**
The Chemical Composition (g $kg^{-1}$ DM) of Dietary Ingredients

| | *Hay* | *Soya bean meal* | *Barley* | *Treated barley* | *Oats* | *Treated oats* |
|---|---|---|---|---|---|---|
| DM (g $kg^{-1}$ fresh weight) | 847 | 870 | 846 | 832 | 862 | 858 |
| Organic matter | 946 | 938 | 978 | 980 | 977 | 979 |
| Digestible organic matter | 610 | — | — | — | — | — |
| Crude protein | 93 | 489 | 113 | 109 | 91 | 91 |
| Total fatty acids | 5·0 | 20·7 | 23·6 | — | 66·9 | — |
| 16:0 | 2·3 | 3·9 | 7·0 | — | 11·4 | — |
| 18:0 | 0·3 | 0·9 | 0·2 | — | 0·7 | — |
| 18:1 | 0·8 | 3·7 | 3·5 | — | 28·0 | — |
| 18:2 | 0·9 | 10·9 | 11·6 | — | 25·6 | — |
| 18:3 | 0·7 | 1·3 | 1·3 | — | 1·2 | — |

The oats also contained a higher proportion of C18 acids, especially 18:1. The fatty acid composition of the formaldehyde-treated cereals was assumed to be the same as that of the respective untreated controls.

Consumption of feeds was essentially complete, and the levels of DM and crude protein intake were close to those planned (Table 2). The ME content of hay was estimated from DOMD content (MAFF 1984). ME intakes calculated using this

**TABLE 2**
Feed and Nutrient Intakes (kg $d^{-1}$) by Animals Given Diets Containing Barley, Treated Barley, Oats or Treated Oats

| | *Barley* | *Treated barley* | *Oats* | *Treated oats* | *SED* |
|---|---|---|---|---|---|
| Hay | 4·24 | 4·23 | 4·23 | 4·22 | |
| Soya bean meal | 1·52 | 1·52 | 1·52 | 1·52 | |
| Barley | 6·76 | 6·66 | — | — | |
| Oats | — | — | 7·32 | 7·15 | |
| Total DM intake | 12·51 | 12·41 | 13·07 | 12·89 | 0·06*** |
| Organic matter | 12·05 | 11·94 | 12·57 | 12·41 | 0·06*** |
| Crude protein | 1·90 | 1·86 | 1·80 | 1·78 | 0·006*** |
| Calculated metabolisable energy (MJ $d^{-1}$)[a] | 144·8 | 143·5 | 145·6 | 143·3 | 0·8 |
| Total fatty acids[b] | 212 | 209 | 542 | 531 | 5*** |
| 16:0 | 63 | 62 | 99 | 97 | 1*** |
| 18:0 | 4 | 4 | 8 | 8 | 0·04*** |
| 18:1 | 33 | 33 | 214 | 209 | 2*** |
| 18:2 | 99 | 98 | 208 | 203 | 2*** |
| 18:3 | 13 | 13 | 14 | 14 | 0·1** |

[a]ME values assumed were: hay, 9·2 MJ $kg^{-1}$ DM (MAFF1984); soya bean meal, 12·3 MJ $kg^{-1}$ DM (MAFF 1984); barley and treated barley, 12·8 MJ $kg^{-1}$ DM (MAFF 1986); oats and treated oats, 12·0 MJ $kg^{-1}$ DM (MAFF 1986).
[b]Fatty acid intakes (g $d^{-1}$) calculated from the fatty acid composition of the fresh cereals prior to formaldehyde treatment.
** $P<0·01$; *** $P<0·001$.

**TABLE 3**
Milk Yield and Composition and the Yields of Milk Constituents for Animals Given Diets Containing Barley, Treated Barley, Oats or Treated Oats

| | *Barley* | *Treated barley* | *Oats* | *Treated oats* | *SED* |
|---|---|---|---|---|---|
| Milk yield (kg $d^{-1}$) | 15·9 | 16·9 | 17·1 | 18·2 | 0·5** |
| Fat (g $kg^{-1}$) | 42·0 | 37·2 | 37·9 | 36·6 | 1·4** |
| Fat (g $d^{-1}$) | 660 | 627 | 649 | 662 | 29 |
| Protein (g $kg^{-1}$) | 33·9 | 33·4 | 31·6 | 31·0 | 0·8** |
| Protein (g $d^{-1}$) | 539 | 560 | 535 | 561 | 16 |
| Lactose (g $kg^{-1}$) | 48·8 | 49·0 | 49·4 | 49·1 | 0·3 |
| Lactose (g $d^{-1}$) | 780 | 831 | 849 | 893 | 29* |

*$P<0·05$; **$P<0·01$.

value and ME contents taken from tables of feedstuff composition (MAFF 1984, 1986) for the remaining feedstuffs were similar for all diets. Intakes of total fatty acids for the oats diets were approximately 2·5 times as great as for the barley diets, and associated with this, and with the differences in fatty acid composition between the cereals, there were substantial differences between diets in the intakes of 16:0, 18:1 and 18:2 acids.

### 3.2 Milk yield and composition

The yield and composition of milk and the yields of milk constituents for animals receiving the experimental diets are shown in Table 3.

Irrespective of the type of cereal included in the diet the formaldehyde treatment tended to increase milk yield ($P>0·05$) and for the barley diet only to reduce milk fat content ($P<0·01$). Milk fat yields were not significantly changed with formaldehyde treatment of the cereals. Formaldehyde treatment was without significant effect on protein and lactose yields with both the barley and oats diets.

As compared with the corresponding diets containing barley, the oats diets were associated with higher ($P<0·05$) milk yields and reduced milk fat contents ($P<0·05$ for the untreated cereals only) such that cereal-related differences in milk fat yields were small and non-significant. Milk protein content was significantly reduced ($P<0·01$) when diets containing oats rather than barley were given, but as these changes were associated with concurrent increases in milk yield, yields of protein were similar for the barley diets and the corresponding oats diets. In line with the effects on milk yield, mean yields of lactose were greater ($P<0·05$) with the oats diets than with the barley diets.

### 3.3 Milk fat composition and fatty acid yields

Formaldehyde treatment of barley or oats had no significant effect on milk fat composition (Table 4) or on the yields of individual fatty acids in milk (Table 5). However, there were marked and significant effects on milk fat composition and on fatty acid yields related to the type of dietary cereal. As compared with corresponding diets containing barley, oats diets led to reductions (generally

**TABLE 4**
Milk Fatty Acid Composition (g $kg^{-1}$ Total Fatty Acids) for Animals Given Diets Containing Barley, Treated Barley, Oats or Treated Oats

| *Fatty acid* | *Barley* | *Treated barley* | *Oats* | *Treated oats* | *SED* |
|---|---|---|---|---|---|
| 6:0 | 20 | 17 | 14 | 16 | 2 |
| 8:0 | 14 | 14 | 10 | 10 | 1*** |
| 10:0 | 35 | 37 | 20 | 21 | 2*** |
| 12:0 | 43 | 45 | 25 | 23 | 3*** |
| 14:0 | 146 | 151 | 104 | 106 | 4*** |
| 16:0 | 395 | 390 | 281 | 275 | 14*** |
| 16:1 | 31 | 29 | 30 | 27 | 2 |
| 18:0 | 101 | 94 | 166 | 161 | 11*** |
| 18:1 *cis* | 171 | 174 | 289 | 301 | 10*** |
| 18:1 *trans* | 10 | 9 | 25 | 22 | 3*** |
| 18:2 | 26 | 30 | 24 | 26 | 1** |
| 18:3 | 9 | 9 | 13 | 12 | 2 |

**$P<0.01$; ***$P<0.001$.

$P<0.001$) in the contents of 6:0–16:0 acids in milk, to increases ($P<0.001$) in 18:0 and 18:1 and to little change in 16:1, 18:2 and 18:3 (Table 4). Associated with these changes there were significant ($P<0.001$) reductions in the yields of 8:0–16:0, significant ($P<0.001$) increases in the yields of 18:0 and 18:1, and no significant changes in the yields of 16:1, 18:2 and 18:3 acids. Increases in both the *cis* and *trans* isomers contributed to the greater secretion of 18:1 observed with oats, but the proportion of *trans* 18:1 in the total 18:1 acids increased only from 0·06 with the barley to 0·08 with the oats diets.

**TABLE 5**
Yields of Individual Milk Fatty Acids (g $d^{-1}$) for Animals Given Diets Containing Barley, Treated Barley, Oats or Treated Oats

| *Fatty acid* | *Barley* | *Treated barley* | *Oats* | *Treated oats* | *SED* |
|---|---|---|---|---|---|
| 6:0 | 12·8 | 10·9 | 8·8 | 10·5 | 1·4 |
| 8:0 | 9·1 | 8·9 | 6·3 | 6·5 | 0·6*** |
| 10:0 | 22·9 | 23·4 | 12·9 | 13·8 | 1·4*** |
| 12:0 | 28·2 | 28·3 | 15·6 | 15·2 | 2·0*** |
| 14:0 | 96·6 | 95·1 | 66·1 | 70·1 | 4·7*** |
| 16:0 | 260·9 | 244·1 | 179·4 | 182·7 | 10·3*** |
| 16:1 | 20·4 | 18·0 | 19·1 | 17·5 | 1·3 |
| 18:0 | 68·2 | 52·2 | 110·5 | 107·4 | 10·8*** |
| 18:1 *cis* | 112·8 | 109·3 | 190·5 | 198·8 | 10·5*** |
| 18:1 *trans* | 6·7 | 5·6 | 16·6 | 14·4 | 2·0*** |
| 18:2 | 17·1 | 19·0 | 15·5 | 17·1 | 1·5 |
| 18:3 | 6·0 | 5·3 | 8·4 | 8·2 | 1·4 |

***$P<0.001$.

## 4 DISCUSSION

### 4.1 Formaldehyde treatment of grain

The tendency for milk yield to increase and the changes in milk fat content which were associated with the formaldehyde treatment of barley and oats in the present experiment with hay-based diets are consistent with those previously reported in heifers receiving silage diets, where the inclusion of treated rather than untreated barley increased milk yield from 14·43 to 15·80 kg $d^{-1}$ and reduced milk fat content from 50·3 to 48·8 g $kg^{-1}$ (Kassem et al 1987). The changes are thought to derive from a slowing of the rate of fermentation of cereal starch and protein in the rumen and an increased passage of protein and starch from the rumen to the small intestine (Kassem *et al* 1987; Van Ramshorst and Thomas 1988).

Despite indications that the cereal starch and protein components were successfully cross-linked by the grain treatment used in the present experiment, the formaldehye treatment conferred no protection against microbial biohydrogenation on the cereal lipids as judged by the effects of the treatment on milk fatty acid composition and fatty acid yields. Estimates for transfer of 18:2 from diet to milk derived using rumen-protected fats ranged from 0.17 to 0.42 (Storry 1981). In the present experiment the oats provided 100 g $d^{-1}$ more 18:2 than the barley, and had formaldehyde treatment reduced ruminal biohydrogenation greater responses in milk 18:2 yield to grain treatment would have been expected for oats than for barley. The close similarity in the yields of 18:2 for all diets strongly suggests that irrespective of formaldehyde treatment dietary polyunsaturated fatty acids were hydrogenated in the rumen. Consistent with that the yields of *trans* 18:1, which is an intermediate in the ruminal biohydrogenation of 18:3 and 18:2 to 18:0 (Christie 1981), were similar for untreated and formaldehyde-treated diets.

### 4.2 Comparison of barley and oats

The diets used here were designed to be isoenergetic, but slight differences between the values adopted for the ME contents of barley and oats and their actual energy contents cannot be ruled out, and an enhanced ME supply may have contributed to the higher milk yield in animals receiving oats as compared with barley diets. None the less, similar responses to oats, although they are not invariably observed (Tommervik and Waldern 1969; Jeffrey *et al* 1976), have been reported elsewhere. Moran (1986) found in cows consuming complete diets containing 600 g $kg^{-1}$ DM as oats or barley that the oats diet led to the production of an additional 2·2 kg $d^{-1}$ of milk, a significant reduction in milk protein content, and, in contrast to the present experiment, an increase in milk fat yield. It appears that under certain circumstances oats, as compared with barley, provide the cow with a mixture of nutrient precursors which favour the secretion of lactose and thus promote milk yield.

In this context two points should be noted. First, Banks and his colleagues (Banks *et al* 1976) observed in cows receiving low-fat diets that fat supplements increased yields of milk and all milk constituents. However, the responses to oats observed in the present experiment are unlikely to be attributable to similar effects arising from the relatively high lipid content of oats; fatty acid intakes with

the control barley diets (~210 g $d^{-1}$) were above the level of ~120 g $d^{-1}$ thought to be adequate for the dairy cow (Banks *et al* 1983). Secondly, there are similarities in effects on milk yield and fat content between oats and formaldehyde-treated barley, and this could indicate that the effects of oats relate to an influence on passage of starch and protein to the small intestine (Van Ramshorst and Thomas 1988).

The reductions in milk protein content observed here with oats are greater than those observed with treated barley. However, this difference in response could be a result of the effect of the oats lipid on milk protein content (Clapperton and Banks 1985).

The effects of replacement of barley with oats on milk fat composition resembled those observed in response to dietary fat supplements containing a high proportion of C18 fatty acids (Storry 1981; Clapperton and Banks 1985). Fat supplements are thought to reduce *de novo* synthesis of 6:0–16:0 within the mammary gland through effects arising from a reduced supply of acetate, because of the influences of the supplementary fats on rumen fermentation, and from inhibition of mammary acetyl-CoA carboxylase (Clapperton and Banks 1985). The concurrent increases in the proportions and yields of 18:0 and 18:1 in milk fat when oats are given are consistent with hydrogenation in the rumen of the additional unsaturated fatty acids supplied in the diet and subsequent intramammary desaturation of the 18:0 formed (Christie 1981). This process has been exploited in the past by adding C18-rich fats to the diet of cows to increase the degree of unsaturation of milk fat (Banks *et al* 1980). The results of the present experiment clearly demonstrate that comparable changes in milk fat composition can be achieved by what is potentially a simple and cheap method, that of including oats in the diet as a replacement for barley.

### 4.3 Implications for human nutrition

Although the evidence for links between dietary fat intake and coronary heart disease remains a matter of debate (NACNE 1983; Committee on Medical Aspects of Food Policy 1984; Brisson 1986), it is probable that the high levels of saturated fat in the UK diet are likely to cause continued concern and adverse criticism. In this experiment it was shown that formaldehyde treatment of barley and oats was ineffective as a means of reducing the biohydrogenation of cereal lipid in the rumen and increasing milk polyunsaturated fatty acid content. However, the feeding of oats in replacement for barley was shown to lead to a substantial reduction in the saturated 6:0–16:0 fatty acid content of milk fat with a concomitant increase in 18:1 especially. Recent human studies have shown that dietary monounsaturated fatty acids were effective in lowering the concentrations of total and low-density lipoprotein cholesterol in blood plasma (Mattson and Grundy 1985), although the effects may be less pronounced than those observed with dietary polyunsaturated fatty acids (Becker *et al* 1983). Also, a diet rich in monounsaturated fatty acids is apparently as effective as a low-fat, high-carbohydrate diet in reducing plasma cholesterol levels (Grundy 1986). Furthermore, monounsaturated fatty acids may be especially beneficial in that, unlike polyunsaturates, they do not reduce plasma levels of high-density lipoprotein

cholesterol, which is thought to be involved in the excretion of cholesterol from the body (Gordon *et al* 1977).

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# Protection against the Toxic Effects of the Favism Factor (Divicine) in Rats by Vitamins E, A and C and Iron Chelating Agents

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*ABSTRACT*

*Five experiments were carried out with weanling Sprague–Dawley rats to determine if prior administration of either free-radical scavenging compounds (vitamins A, C and E) or chelating agents (EDTA and desferoxamine) affected the toxicity of divicine (DV). In all experiments, intraperitoneal (IP) injections of 250 mg $g^{-1}$ body weight of DV alone resulted in 100% mortalities within 24 h with most of the deaths occurring before 4 h. Death was accompanied by a rapid decrease in the concentration of glutathione (GSH) in the red blood cells (RBC) and the 576 nm/628 nm absorbance ratio of haemoglobin. The first experiment demonstrated that IP injections of different amounts of vitamin E, 1 h prior to DV injection, prevented the decrease in the haemoglobin absorbancy ratio and GSH concentration and greatly reduced mortalities. In rats that received 1000 IU of vitamin E $kg^{-1}$ body weight prior to DV injection, mortality was only 20%. The second experiment demonstrated that the optimal time for IP administration of vitamin E was 1 to 4 h prior to DV injection although some protection was obtained after 96 h. In contrast, the optimal time for intramuscular injections was 24 h prior to DV administration. A dosage of 250 or 500 IU of vitamin E $kg^{-1}$ body weight provided complete protection (zero mortalities) against the toxic effects of DV. In the third experiment, the addition of varying amounts of vitamin E to the diet resulted in a dose-dependent mortality curve with no deaths occurring in rats fed diets containing the highest concentrations of vitamin E. The fourth experiment also demonstrated that vitamin A, vitamin C, EDTA and desferoxamine each*

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*protected rats to varying degrees against the toxic effects of DV. In the final experiment it appeared that the combined injections of a free-radical scavenging compound (vitamin E) and a metal chelator (desferoxamine) provide more protection than vitamin E alone. These results demonstrate for the first time* in vivo *that certain vitamins, especially vitamin E, and metal chelators can provide varying and in some cases 100% protection against the toxic effects of DV.*

*Key words*: Divicine, vitamin E, interaction, toxic effects, favism, chelators.

## 1 INTRODUCTION

The consumption of faba beans has been shown to cause favism, a haemolytic anaemia, in glucose-6-phosphate dehydrogenase (G6PD) deficient humans (Belsey 1973; Mager *et al* 1980), and egg size depression in laying hens (Campbell *et al* 1980). The causative agents are divicine (DV) and isouramil, which are highly reactive compounds generating free radicals and catalysing a one-electron oxidation/reduction shuttle between reducing compounds and molecular oxygen (Mager *et al* 1965; Lin *et al* 1977; Mager *et al* 1980; Arese *et al* 1981; Chevion *et al* 1982; Albano *et al* 1984; Baker *et al* 1984; Yannai and Marquardt 1985; Arbid and Marquardt 1986, Winterbourn *et al* 1986). These compounds are produced in the gastrointestinal tract, following the hydrolysis by the microflora of the parent compounds, vicine and convicine, (Frohlich and Marquardt 1983; Hegazy and Marquardt 1984) and are absorbed by the blood. They then interact with the abundant supply of oxygen in the blood to produce superoxide radicals (Albano *et al* 1984; Hegazy and Marquardt 1984) which, if not neutralised by the free-radical scavenging system, cause cell damage. The oxygen- and iron-rich red blood cells (RBC) are a particularly good target that can be irreversibly damaged under the appropriate conditions. This may lead to favism-like signs.

Indirect evidence would suggest that certain antioxidants can protect against the toxic effects of these compounds. Corash *et al* (1980, 1982) reported that vitamin E supplementation of G6PD deficient individuals decreased chronic haemolysis. Vitamin E deficiency, which occurs in certain pathological conditions, is accompanied by increased concentrations of malondialdehyde (*in vitro*) and increased peroxidative damage (*in vivo*), two factors responsible for shortened red cell survival times. These haematological abnormalities are also alleviated by vitamin E supplementation (Chiu *et al* 1982). Studies with the laying hen (Muduuli *et al* 1982) demonstrated that the vicine-induced depressions in fertility and in hatchability of eggs were greatly reduced with moderate levels of vitamin E supplementation. In addition the iron chelating agent desferoxamine has been shown to block the antimalarial and haemolytic activity of DV (Clark and Cowden 1985). The transition metal chelating agents have also been shown to provide complete protection, *in vitro*, against enzyme inactivation caused by favism-inducing agents and the transition metals, presumably by blocking a transition metal iron-driven Fenton-type reaction (Navok and Chevion 1984).

The above studies would suggest that vitamin E or the tocopherols and probably other free-radical scavengers such as vitamin C, flavonoids, $\beta$-carotene, ureate and plasma protein would quench the free radicals produced by DV and isouramil and thereby neutralise their effects (Chiu *et al* 1982; Sies 1985). This has not been investigated directly in humans for ethical reasons or in animals because suitable models have not been available. Also the supply of the aglycones has been limited. Yannai and Marquardt (1985) and Arbid and Marquardt (1986) have recently demonstrated that the rat can be sensitised to the effect of vicine, convicine, DV or isouramil following IP injections or intravenous (IV) injections of the latter two compounds. The induced signs are similar in many respects to those observed in favism in humans, which include a dramatically reduced concentration of glutathione (GSH) and an altered ultraviolet absorbance pattern in haemolysed blood (Arbid and Marquardt 1986). This is indicative of the formation of the ferryl species (a haemoglobin–peroxide complex) of haemoglobin. (Winterbourn *et al* 1986). Other signs were an increased respiration rate, generalised cyanosis and, after several hours, death apparently caused by asphyxiation (Yannai and Marquardt 1985; Arbid and Marquardt 1986). The weight of splenic tissue and per cent haematocrit decreased 24 h after DV administration indicating that the damaged erythrocytes were being removed by the reticuloendothelial system.

The objectives of the present work were to determine if certain free-radical scavenging compounds such as vitamins E, A and C, and the iron chelating agents, desferoxamine and EDTA, could protect the rat against the toxic effects of DV. Factors investigated were: route of administration (oral, IP, IM); effective dosage of the protective compounds; duration of the protective effect; and possible interaction between a free-radical scavenger and a metal chelating agent. Parameters measured were: mortalities, haematocrit, blood GSH concentration, and ratio of haemoglobin absorbance at 576 nm to absorbance at 628 nm.

## 2 MATERIALS AND METHODS

### 2.1 Source of chemicals and preparation of compounds for injection into rats

Vitamin E as DL-$\alpha$-tocopherol with a potency of 1 IU $mg^{-1}$, and synthetic all-*trans* vitamin A palmitate in corn oil at a potency of 1 000 000 IU $g^{-1}$, were obtained from United States Biochem Corp, Cleveland, Ohio. Vitamin C, as the anhydrous sodium salt of ascorbic acid, was from Sigma Chemical Co, St Louis, MO; EDTA, as the disodium salt, was from Fisher Scientific, Winnipeg, Manitoba; and desferoxamine mesylate (Desferal) was from CIBA-Geigy Canada Ltd, Mississauga, Ontario.

Pure vicine was prepared as described by Marquardt *et al* (1983) and divicine by acid hydrolysis of vicine as described by Arbid and Marquardt (1986). Other chemicals were from Sigma Chemical Co (St Louis, MO) or Fisher Scientific (Winnipeg, MB).

DV was prepared as a suspension in degassed PBS (0·05 M phosphate buffer, pH 7·4, 0·075 M with respect to NaCl) at 26°C just prior to injection. Very little oxidation of DV occurred when treated in this manner. In general the concentra-

tion of DV was 25 mg (0·18 mmol) $ml^{-1}$ although in Experiment 5 it was 35 mg $ml^{-1}$. PBS (1 ml $kg^{-1}$ body weight) plus DV or PBS (control) were injected IP so that 0, 250 or 350 mg DV $kg^{-1}$ body weight was delivered. The middle dosage was used for most studies as it was shown in a previous study to cause 100% deaths (Arbid and Marquardt 1986). Vitamins E and A were dissolved in corn oil, and vitamin C, EDTA and desferoxamine in PBS. The total volume of vitamin or chelating agent solution injected into rats was 10 ml $kg^{-1}$ body weight. The amounts of the active principles delivered are indicated below. Control rats received the same volume of vehicle.

### 2.2 Rat experiments

Young male albino rats of the Sprague–Dawley strain from the University colony were maintained on a commercial diet (Wayne Feed Division, Continental Grain Co, Chicago, IL) containing 24% protein and tap water. The diet was estimated to contain an equivalent of 30 mg (30 IU) of DL-$\alpha$-tocopherol acetate $kg^{-1}$ diet. Other nutrients were at recommended levels (National Research Council 1978). Rats for each experiment, except for Experiment 3, averaged ~104±4 g±SE. The rats were randomly divided into treatment groups of five each. All experiments were duplicated.

In Experiment 1, five rats from each treatment were injected IP with 10 ml $kg^{-1}$ body weight of either vitamin E in the amounts indicated in Table 1 or corn oil. After 1 h, PBS or DV in PBS (10 ml $kg^{-1}$ body weight) was injected IP into the rats as indicated in Table 1. Blood was collected from the retro-orbital venous plexes (Helperin *et al* 1951) in heparinised tubes 1 h after DV injection, mixed gently and kept on ice. GSH, haematocrit and haemoglobin absorbancy ratio (576 nm/628 nm×100) were immediately determined. Rats were checked for mortalities at 1, 4 and 24 h after DV injection.

**TABLE 1**

Effects of IP Injections of Vitamin E on the Toxicity of Subsequently Injected DV[a]

| *Vitamin E injected, IU $kg^{-1}$ body weight* | *DV mg $kg^{-1}$ body weight* | *Cumulative mortalities* | | | *Haematocrit at 1 h* | *GSH at 1 h, mg $litre^{-1}$ blood* | *Haemoglobin absorbance ratio at 1 h, 576 nm/628 nm ×100* |
|---|---|---|---|---|---|---|---|
| | | *1 h* | *4 h* | *24 h* | | | |
| | | *%* | | | *%* | | |
| 0 | 0 | $0^{c}$ | $0^{d}$ | $0^{d}$ | $36^{c}$ | $240^{bc}$ | $83^{b}$ |
| 1000 | 0 | $0^{c}$ | $0^{d}$ | $0^{d}$ | $46^{b}$ | $270^{b}$ | $74^{b}$ |
| 1000 | 250 | $0^{c}$ | $10^{cd}$ | $20^{c}$ | $47^{b}$ | $240^{bc}$ | $99^{b}$ |
| 500 | 250 | $10^{c}$ | $20^{cd}$ | $20^{c}$ | — | $240^{bc}$ | — |
| 250 | 250 | $10^{c}$ | $30^{c}$ | $30^{c}$ | $44^{b}$ | $200^{c}$ | $57^{b}$ |
| 0 | 250 | $60^{b}$ | $100^{b}$ | $100^{b}$ | $42^{b}$ | $70^{d}$ | $6^{c}$ |
| SE | | 8 | 13 | 12 | 1 | 20 | 10 |

[a]See Section 2 for experimental detail. Means in each column not showing a common letter differ significantly ($P<0·05$). The coefficient of correlations ($r$) between 24 h mortalities and GSH concentration was −0·98, ($P<0·05$), between 24 h mortalities and haemoglobin absorbance ratio −0·93 ($P<0·05$) and between GSH concentration and haemoglobin absorbance ratio 0·94 ($P<0·05$). Mortalities were not correlated with haematocrit values ($P>0·05$).

**TABLE 2**
Effect of Time and Route of Administration on Efficacy of Vitamin E as a Protective Agent against DV Toxicity[a]

| *Vitamin E* | | *DV injected* | *Mortalities at 24 h* | | *GSH at 1 h* | |
|---|---|---|---|---|---|---|
| *Amount injected IU kg$^{-1}$* | *Time prior to DV injected h* | *mg kg$^{-1}$* | *IP %* | *IM %* | *IP mg litre$^{-1}$* | *IM blood* |
| 0 | 0 | 0 | 0$^{e}$ | 0$^{d}$ | 250$^{b}$ | 250$^{b}$ |
| 0 | 0 | 250 | 100$^{b}$ | 100$^{b}$ | 40$^{e}$ | 40$^{f}$ |
| 500 | 1 | 250 | 20$^{de}$ | 70$^{c}$ | 170$^{c}$ | 100$^{de}$ |
| 500 | 4 | 250 | 10$^{de}$ | 50$^{c}$ | 170$^{c}$ | 80$^{def}$ |
| 500 | 24 | 250 | 35$^{cd}$ | 0$^{d}$ | 140$^{cd}$ | 210$^{c}$ |
| 500 | 48 | 250 | 60$^{c}$ | 20$^{d}$ | 60$^{e}$ | 80$^{def}$ |
| 500 | 96 | 250 | 60$^{c}$ | 50$^{c}$ | 100$^{cd}$ | 120$^{d}$ |
| SE | | | 9 | 9 | 10 | 10 |

[a]See Section 2 for experimental detail. Means in each column not showing a common letter differ significantly ($P<0{\cdot}05$). The coefficient of correlation ($r$) between mortalities and GSH concentration was $-0{\cdot}91$ ($P<0{\cdot}001$) for IP and $-0{\cdot}83$ ($P<0{\cdot}01$) for IM injections.

In Experiment 2, five rats from each treatment group were injected IP or IM with vitamin E or corn oil at time intervals of 0, 1, 4, 24, 48 or 96 h prior to IP injections of DV. Blood was collected 1 h after DV injection following the procedure used in Experiment 1. Treatments and amounts of compounds injected are outlined in Table 2 and Fig 1. Blood was assayed for GSH, and mortalities were recorded 24 h after DV injection.

In Experiment 3, rats were randomly divided into five groups of five rats each, fed the commercial diet that had been finely ground and supplemented with vitamin E at the concentrations given in Table 3. The diets and water were fed *ad libitum* for 7 days and each group was injected with 10 ml kg$^{-1}$ body weight of DV or PBS as shown in Table 3. Blood was collected 1 h after DV injection and analysed as in Experiment 1. Mortalities were recorded 24 h after DV injection.

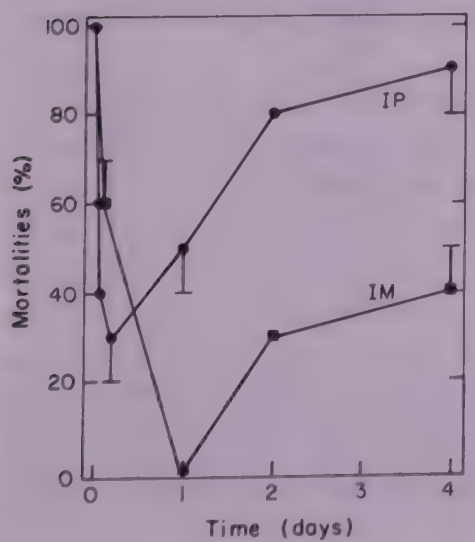


**Fig 1.** Mortalities in DV-injected rats (25 mg kg$^{-1}$ body weight) that had been previously injected either IP or IM with 250 IU of vitamin E kg$^{-1}$ over different time periods. The abscissa indicates time of DV injections after vitamin E injections. Means with no SE bars were too small to see on the graph. See Section 2.2 for further details.

**TABLE 3**
Effect of Dietary Vitamin E on the Toxicity of IP-injected DV[a]

| *Vitamin E added to diet IU kg$^{-1}$* | *Final body weight[b] g* | *DV mg kg$^{-1}$* | *Mortalities at 24 h, %* | *Haematocrit at 1 h, %* | *GSH at 1 h, mg litre$^{-1}$* | *Haemoglobin absorbancy ratio at 1 h, 576 nm/628 nm ×100* |
|---|---|---|---|---|---|---|
| 300 | 137 | 250 | 100$^{c}$ | 48$^{c}$ | 20$^{e}$ | 19$^{e}$ |
| 7500 | 135 | 250 | 30$^{d}$ | 48$^{c}$ | 140$^{d}$ | 53$^{d}$ |
| 37500 | 139 | 250 | 20$^{d}$ | 45$^{d}$ | 150$^{d}$ | 68$^{cd}$ |
| 75000 | 128 | 250 | 0$^{e}$ | 41$^{e}$ | 220$^{c}$ | 68$^{cd}$ |
| 300 | 142 | 0 | 0$^{e}$ | 37$^{f}$ | 230$^{c}$ | 88$^{c}$ |
| SE | | | 12 | 1 | 17 | 6 |

[a]Means in each column not showing a common letter differ significantly ($P<0{\cdot}05$). The coefficients of correlation ($r$) were: mortalities×GSH, −0·98 ($P<0{\cdot}005$); mortalities×haemoglobin absorbance ratio, −0·95 ($P<0{\cdot}05$) haemoglobin absorbance ratio×GSH, 0·95 ($P<0{\cdot}05$). Other comparisons were not correlated ($P>0{\cdot}05$).
[b]The average feed intake was 300 g per treatment group.

**TABLE 4**
Effect of IP-injected Vitamins A and C, EDTA and Desferoxamine on the Toxicity of IP-injected DV[a]

| *Compound* | *Dosage Units kg$^{-1}$* | *DV mg kg$^{-1}$* | *Mortalities at 24 h %* | *GSH at 1 h mg kg$^{-1}$* | *Haemoglobin absorbancy ratio at 1 h, 576 nm/628 nm ×100* |
|---|---|---|---|---|---|
| Control | Saline | 0 | 0$^{e}$ | 240$^{bc}$ | 83$^{b}$ |
| | Saline | 250 | 100$^{b}$ | 70$^{h}$ | 6$^{d}$ |
| Vitamin A | 1·0 | 0 | 0$^{e}$ | 220$^{bcd}$ | 67$^{b}$ |
| ($10^{6}$ IU) | 1·0 | 250 | 20$^{e}$ | 170$^{def}$ | 39$^{c}$ |
| | 0·5 | 250 | 50$^{d}$ | 170$^{def}$ | 8$^{d}$ |
| Vitamin C | 5·0 | 0 | 0$^{e}$ | 200$^{cde}$ | 72$^{b}$ |
| (mmol) | 5·0 | 250 | 60$^{cd}$ | 80$^{h}$ | 11$^{d}$ |
| | 2·5 | 250 | 70$^{cd}$ | 110$^{fgh}$ | 9$^{d}$ |
| EDTA | 1·3 | 0 | 0$^{e}$ | 270$^{b}$ | 86$^{b}$ |
| (mmol) | 1·3 | 250 | 80$^{bc}$ | 160$^{ef}$ | 8$^{d}$ |
| | 0·7 | 250 | 80$^{bc}$ | 100$^{fgh}$ | 8$^{d}$ |
| Desferoxamine | 1·0 | 0 | 0$^{e}$ | 200$^{cde}$ | 73$^{b}$ |
| (g) | 1·0 | 250 | 50$^{d}$ | 90$^{gh}$ | 19$^{cd}$ |
| | 0·5 | 250 | 60$^{cd}$ | 80$^{h}$ | 10$^{d}$ |
| SE | | | 7 | 10 | 5 |

[a]See Section 2 for experimental detail. Means in columns not showing a common letter differ significantly ($P<0{\cdot}05$). The coefficient of correlation ($r$) between mortalities and GSH concentration, mortalities and haemoglobin absorbancy ratio, and GSH and haemoglobin absorbancy ratio were −0·80 ($P<0{\cdot}001$), −0·93 ($P<0{\cdot}0001$) and 0·83 ($P<0{\cdot}001$), respectively.

The procedure for Experiment 4 was the same as that for Experiment 1 except that the compounds injected in IP in PBS were vitamin A in corn oil, and vitamin C, EDTA and desferoxamine. Only corn oil was injected into the controls as previous results had demonstrated that there were no differences in any of the parameters when no vehicle, oil or PBS, were injected. The total amounts of the different compounds that were injected are given in Table 4.

The procedures for Experiment 5 were the same as for Experiment 1 except that combinations of vitamin E and desferoxamine were injected 1 h prior to DV injection. Again only corn oil controls were included. The dosage of DV was also greater than that of Experiments 1–4, being 350 mg $kg^{-1}$ body weight.

### 2.3 Other analyses

Haematocrit was determined using standard procedures (Arbid and Marquardt 1986), the absorbance ratio of haemolysed blood as described previously (Arbid and Marquardt 1986), and GSH according to the procedure of Beutler *et al* (1963). The data were subjected to analysis of variance, and in cases where the effects of treatments were found to be significant the least significance difference test was performed (Snedecor and Cochran 1980).

## 3 RESULTS AND DISCUSSION

### 3.1 Influence of IP injections of vitamin E on DV toxicity

Although none of the control rats, or those injected only with the high dosage of vitamin E, died, 100% of those injected with 250 mg DV $kg^{-1}$ body weight died (Table 1). However, vitamin E, when injected 1 h prior to DV, dramatically reduced mortalities, with the maximum being 30% in rats given 250 IU of vitamin E $kg^{-1}$. There did not appear to be a dose response ($P>0{\cdot}05$), suggesting that the low vitamin E dosage provided near maximal protection. Most of the deaths following DV administration occurred within 1 h and all within 4 h. The time-course pattern for deaths in all subsequent experiments was similar but not identical as some additional rats died after 4 h. As a result only 24-h data are presented henceforward. A similar time course for death occurred in a previous study in which only vicine or convicine was administered (Arbid and Marquardt 1986).

Haematocrit values for blood collected 1 h after DV administration increased ($P<0{\cdot}05$), which may be attributed to haemoconcentration caused by the movement of body fluids into the gastrointestinal tract (Arbid and Marquardt 1986). Blood samples were not collected after 24 h in the work reported here as all rats treated with only DV died. However, in previous studies it was shown that when a smaller dose of DV was injected into rats, haematocrits either increased or were not affected within 1·5 h of DV administration; after 24 h there was a dramatic decrease in haematocrit concentration which corresponded with the increase in the weight of the spleen (Yannai and Marquardt 1985; Arbid and Marquardt 1986). Presumably splenic entrapment of DV-damaged erythrocytes occurred and this was a time-dependent process. A similar pattern would probably have

occurred in the current series of experiments if samples could have been taken after 24 h.

Both GSH and the haemoglobin absorbance ratio were greatly depressed ($P<0.05$) by DV treatment alone which was similar to results obtained previously both *in vivo* and *in vitro* (Yannai and Marquardt 1985; Arbid and Marquardt 1986). In general DV-treated RBC have increased absorbance values above 600 nm compared with untreated RBC (Arbid and Marquardt 1986). Winterbourn *et al* (1986) also demonstrated that similar spectral changes occur when DV is added to purified oxyhaemoglobin and that these are identical to those seen when $H_2O_2$ is added to oxyhaemoglobin. They concluded that DV causes the formation of a haemoglobin/peroxide complex and that a ferryl species of haemoglobin was formed.

It has been well documented that DV and isouramil decrease GSH concentrations both *in vitro* (Mager *et al* 1965, 1980; Arese *et al* 1981) and *in vivo* (Yannai and Marquardt 1985; Arbid and Marquardt 1986), and that the symptoms of favism in humans are closely associated with the depletion of GSH (Mager *et al* 1965, 1980). On the basis of previous studies with rats (Yannai and Marquardt 1986; Arbid and Marquardt 1986) and other *in vitro* studies (Mager *et al* 1965, 1980; Arese *et al* 1981; Baker et al 1984) it appears that DV first causes a reduction of GSH concentration in the RBC. If the dosage is sufficiently high, there is near complete depletion of GSH which is then followed by peroxidative changes in the haemoglobin and other components of the RBC. The cell then loses its functional capacity to exchange respiratory gases together with its rheological properties, and as a result death occurs due to asphyxiation.

However, the administration of vitamin E prior to DV not only reduced mortalities but also prevented the associated decreases in GSH concentration in the blood and the haemoglobin absorbancy ratio. Presumably vitamin E treatment interferes with DV by acting as a free radical scavenger (Chiu *et al* 1982; Sies 1985), thereby preventing GSH depletion and the subsequent peroxidative effects. The net effect is that vitamin E reduces DV toxicity.

### 3.2 Time and route of vitamin E administration

Table 2 and Fig 1 demonstrate that the efficacy of vitamin E as a protective agent against the toxic effects of DV is influenced by the time between vitamin E and DV administration and by the route of administration. In general the patterns of response for rats administered either 500 (Table 1) or 250 IU (Fig 1) of vitamin E $kg^{-1}$ body weight were similar. IP administration of vitamin E provided the most rapid protection against the toxic effects of DV, with minimal mortalities occurring between 1 and 4 h. Thereafter the protective effect of vitamin E was gradually lost and by day 4 there was very little residual effect with the low vitamin E group but considerably more with the high vitamin E treatment group. The pattern with the IM-injected rats was somewhat different from that in the IP-treated groups as the protective effect of vitamin E was considerably delayed, with maximal protection (zero deaths in both treatment groups) being obtained after 24 h. Thereafter there was a gradual increase in mortalities in both groups. The overall mortality for rats administered vitamin E in this study was 42%, which compares with a

death rate of 100% in rats receiving only DV. No deaths occurred in animals administered only vitamin E and PBS.

The high negative correlations (footnotes of Table 1 and 2) between mortalities and the concentration of GSH in RBC and between mortalities and the haemoglobin absorbancy ratio, and the high positive correlations between GSH concentrations and the haemoglobin absorbancy ratio, indicate that all three effects are closely interrelated, probably in a cause-and-effect relationship as described previously.

Vitamin E when added to the diet was also equally effective at protecting rats against the deleterious effects of DV (Table 3). There was a dose–response effect of vitamin E with the highest dosages being most protective. There were no mortalities in any of the DV-treated rats that had been fed diets containing the high dosages of vitamin E, whereas mortalities were 100% in those fed the diet that contained only the recommended daily amount of vitamin E. Haematocrit values were similar to those in Experiment 1 and would suggest that within 1 h of DV administration haemoconcentration occurred which was probably caused by the movement of fluid from the blood into the gastrointestinal tract. GSH concentration and haemoglobin absorbancy ratio were also negatively correlated with mortalities (see Table 1 footnotes). In addition the patterns of change of GSH, haemoglobin absorbancy ratio and haematocrit were the same as those observed in the previous experiments.

This is the first demonstration that vitamin E can prevent mortalities and certain associated metabolic changes in animals injected with DV, although other, indirect, research has indicated that this should be possible. As indicated in the Introduction, a positive response has been reported in egg fertility to vitamin E supplementation in the diet of vicine-fed laying hens (Muduuli *et al* 1982), in G6PD deficient individuals with chronic haemolysis (Corash *et al* 1980, 1982), and in individuals with increased peroxidative damage to RBC associated with certain pathological conditions (Chiu *et al* 1982). The last observation suggests that vitamin E status can influence the toxicity of peroxidising compounds which would include DV.

The results here also demonstrated that (a) vitamin E can provide almost complete protection against the toxic effects of DV; (b) vitamin E is equally efficacious when administered orally, IP or IM; (c) the degree of protection is influenced by the dosage of vitamin E administered and by the time between a single injection of vitamin E and DV.

### 3.3 Protective effects of other free radical scavengers, metal chelators and their interaction

Different concentrations of vitamins A and C, desferoxamine (an iron chelator), and possibly EDTA, when injected IP into rats 1 h before DV injection provided protection against DV toxicity, albeit to varying degrees (Table 4). Of the compounds tested, vitamin A was most protective, and the response was dose dependent. Only 20% of the DV-treated rats died when 100000 units of vitamin A activity was administered to rats in contrast with 100% mortality of the rats treated with DV alone. Vitamin C also afforded considerable protection, which is

**TABLE 5**
Synergistic Effect of IP-injected Vitamin E and Desferoxamine on DV Toxicity[a]

| *Vitamin E IU kg$^{-1}$* | *Desferoxamine g kg$^{-1}$* | *DV mg kg$^{-1}$* | *Mortalities at 24 h %* | *Haematocrit at 1 h %* | *GSH at 1 h mg litre$^{-1}$* | *Haemoglobin absorbancy ratio at 1 h 576 nm/628 nm ×100* |
|---|---|---|---|---|---|---|
| 0 | 1·0 | 350 | 100$^{b}$ | 43$^{d}$ | 43$^{d}$ | 5·7$^{c}$ |
| 500 | 0 | 350 | 90$^{bc}$ | 56$^{b}$ | 55$^{cd}$ | 7·6$^{c}$ |
| 500 | 1·0 | 350 | 80$^{bc}$ | 51$^{bc}$ | 80$^{c}$ | 7·7$^{c}$ |
| 500 | 0·5 | 350 | 70$^{c}$ | 49$^{c}$ | 114$^{b}$ | 17·6$^{b}$ |
| SE | | | 5 | 1 | 8 | 2·0 |

[a]See Section 2 for experimental detail. Means in columns not showing a common letter differ significantly ($P<0·05$). The coefficient of correlations ($r$) was: $-0·80$ ($P<0·05$) between mortalities and GSH, and $-0·71$ ($P<0·05$) between mortalities and haemoglobin absorbancy ratio. Other correlations were not significant ($P>0·05$).

surprising in view of previous *in vitro* results which showed that ascorbic acid enhances rather than suppresses the toxicity of DV (Mager *et al* 1965, 1980; Arese *et al* 1981). Presumably effects *in vivo* and *in vitro* are different. Vitamin C, nevertheless, is considered to be a free-radical scavenger (Sies 1985), and it would be expected to scavenge the superoxide radicals or other free radicals that are formed when oxygen reacts with DV (Chevion 1982; Albano 1984). The mode of action may be similar to that obtained with vitamins A and E. Additional research is needed to establish the reason for the apparent discrepancies between *in vitro* and *in vivo* studies.

Desferoxamine, an iron chelator, also reduced the toxicity of DV but there did not appear to be a dose–response effect. This suggests that lower doses may be equally effective. EDTA did not provide any significant protection ($P>0·05$). *In vitro* studies have demonstrated that desferoxamine is also able to block the haemolytic activity of DV (Clark and Cowden 1985). In addition, transition metal chelating agents have been shown to provide complete protection *in vitro* against enzyme inactivation caused by favism-inducing agents such as DV and the transition metals (Navok and Chevion 1984), presumably by blocking a transition metal ion driven Fenton-type reaction (Chiu *et al* 1982). A similar type of effect may also occur *in vivo*. In contrast, Meloni *et al* (1986) reported that desferoxamine was unsuccessful in the treatment of favism in three humans. This and other free-radical scavenging compounds, however, may only be effective if administered prior to, but not after, ingestion of the toxic favism-producing compounds. In general the pattern of changes in the haematocrit, GSH concentration, the haemoglobin absorbancy ratio and mortalities in the current experiment was in agreement with the mortality data and the results from the previous experiments.

The final study attempted to establish if there was an interaction between a metal chelating agent and a free radical scavenging compound in the toxicity of DV. The results (Table 5) indicate that a combination of vitamin E and desferoxamine offered more protection against DV than vitamin E alone.

## 4 CONCLUSIONS

This paper provides direct evidence *in vivo* that free-radical scavenging compounds, specifically vitamins A, C and E, and metal chelating compounds (desferoxamine and possibly EDTA) can protect the rat against DV-induced death and associated biochemical changes. The effects of DV in the rat are analogous in many respects to those of favism in humans (Yannai and Marquardt 1985; Arbid and Marquardt 1986).

In addition to the protective effects of the above compounds, other dietary components may act synergistically or additively to increase protection against DV or isouramil or may, in fact, have an opposite effect. This would depend on whether they promote the generation or scavenging of free radicals. It may, nevertheless, be concluded that certain compounds may be capable of greatly modifying individual susceptibility to DV and, presumably, to favism. These may be one of the important additional factor(s) proposed by Mager *et al* (1980), along with G6PD deficiency and exposure to faba beans, which influence the degree and severity of the haemolytic attack. A better understanding of the interactions among these factors may assist in clarifying the bizarre and rather unpredictable mode of occurrence of the disease in susceptible individuals.

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# Carcinogenic Polycyclic Aromatic Hydrocarbons (PAH) Determined in Nigerian Kundi (Smoke-dried Meat)

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*ABSTRACT*

*Chemical analyses of kundi show that, apart from a high level of carcinogenic benzo(a)pyrene (10·5–66·9 ng $g^{-1}$), eight other polycyclic aromatic hydrocarbons (PAH) are present at various concentrations. The high levels of PAH in kundi are due to the high glow and smoking temperatures averaging 926°C and 191·5°C, respectively. Column chromatography was used for PAH extraction with propylene carbonate as the eluting chemical; thin layer chromatography (TLC) on acetylated cellulose layer plates was used for separation, and the determination was performed using spectrophotofluorimetry. The public health implications of PAH as one of the possible carcinogenic factors in the high incidence of primary liver and stomach cancer reported in Nigeria are highlighted.*

*Key words*: Carcinogenic, polycyclic aromatic hydrocarbons, benzo(*a*)-pyrene, smoking, kundi, smoke-dried meat, preservation, chromatography, spectrophotofluorimetry.

## 1 INTRODUCTION

There is a high demand for meat in Africa but production falls short of demand (ECA 1974) and Nigerians consume a paltry 4·0 g per caput per day; fish consumption is 8·2 g per caput per day (NCGR 1981). Therefore it is necessary to preserve the little meat available by preventing its spoilage and contamination. Smoke preservation of meats is particularly useful in the developing countries where the cost and the technology to maintain energy-consuming preservation methods are beyond reach (Mann 1960; Owen *et al* 1979). Unfortunately, this



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age-long method of smoking for the purpose of preservation, with consequent flavour and colour attractions, has been found to produce meats containing some undesirable chemicals, notably, benzo(*a*)pyrene, a proven carcinogen (IARC 1973). Smoke, which is the result of destructive anaerobic distillation of wood followed by partial oxidation, is the source of the PAH in smoke-dried meats (Potthast 1978). Hamm (1977) detected 27 PAH in smoke condensates, and Potthast (1979) reported the presence of 64 PAH in smoke and smoked meat products.

The presence and quantity of PAH in smoked meats increase in proportion to the temperature of treatment (ie the glow and smoking temperatures) and closeness to the source of heat, suggesting pyrolysis of wood and the meat fat as the origin (Potthast 1978). However, when mutton was singed with coal, benzopyrenes were found, whereas none was detected when propane gas was used (Potthast 1979).

Icelandic smoked fish and meats have been found to contain high levels of PAH (Bailey and Dungal 1958; Thernsteinsson 1969), as do Nigerian traditionally smoked meat and fish where a range of 11·1–66·9 ng $g^{-1}$ benzo(*a*)pyrene levels in traditionally smoked fish (Afolabi *et al* 1983) and 8·5 ng $g^{-1}$ benzo(*a*)pyrene in suya meat (spiced skewered beef pieces grilled over an open fire and not intended for long-term storage) were reported (Bababunmi *et al* 1982). Thirteen of the food PAH are proven carcinogens (IARC 1973), and five of the proven carcinogenic PAH are capable of inducing leukaemia, forestomach tumours, hepatoma and pulmonary adenoma when administered orally (Adelman and Suess 1970).

The determination of PAH in foods is very cumbersome and time consuming. Organic solvents are normally used to extract PAH from foods with subsequent separation by column chromatography using solvents of different polarity (Tracor/Jitco Inc 1973). A method involving methanol/water/cyclohexane extraction, acetone concentration, and identification and determination by gas chromatography was developed by Grimmer and Böhnke (1975) and later accepted as the officially recommended method (joint IUPAC–AOAC commission on food additives) for the determination of PAH in foods (Haenni 1978). The method used for acetone concentration (modified after Grimmer and Böhnke 1975 and Potthast and Eigner 1975) involved the extraction of the PAH in 15 ml cyclohexane and careful evaporation to 1 ml thereby concentrating the PAH in 1 ml cyclohexane. The concentrated extract was used for PAH separation on thin-layer chromatography.

In this work, carcinogenic benzo(*a*)pyrene and other PAH contaminating Nigeria's smoke-dried meat, known locally as kundi, are determined. The public health hazards of consuming such meat are highlighted.

## 2 MATERIALS AND METHODS

The smoked meat was the traditionally oven smoke-dried meats, usually beef, called kundi. Kundi is a very dry, darkly smoked, boneless piece of meat weighing between 15 and 75 g. Fresh meat pieces were smoked over half-drum ovens, and

the fire glow and meat chamber smoking temperatures were taken using a Ni–Cr–Ni thermocouple (Therm model 2210-6 by Therm, West Germany). The former temperatures were taken by dipping the thermocouple into the burning wood fire and the latter by touching the meat pieces on the top of the half-drum oven with the thermocouple.

The method of Potthast and Eigner (1975) was used with some modifications. The pieces of kundi were frozen in liquid nitrogen in a vacuum flask, and were then broken into pieces using a mortar and pestle and minced finely using a kitchen grinder (Moulinex, France). About 20 g minced meat was soaked in boiling water for 15 min. The meat was then thoroughly mixed with 50 g sodium sulphate plus 25 ml butan-2-one, 25 ml cyclohexane and 50 g celite. The solvents were evaporated in a vacuum drying cabinet at 40°C for 2 h. A chromatography column was set up with 5 cm celite above the glass frit. The dry material from the drying cabinet was poured inside the column and covered with a 2-cm layer of sea sand. The PAH were then extracted from the sample using 200 ml propylene carbonate. About 170–200 ml eluate was collected and poured into a 2-litre separating funnel, 700 ml distilled water was added followed by 300 ml 5 M NaOH, and the alkaline aqueous solution of propylene glycol which separated was shaken with four 200 ml portions of distilled benzene. The benzene extract, which contained the PAH, was dried by pouring through a mixture of 70 g calcium chloride and 30 g celite. The extract was finally cleaned up by passing the extract through a layer of activated Florisil. The extract was taken to dryness, dissolved in cyclohexane and concentrated to 1·0 ml by weight (1·0 ml cyclohexane weighs 0·78 g). This is the concentration for separation on the TLC acetylated cellulose layer plate. The TLC plate was divided into 2·3 cm-wide portions. A standard, PAH solution (0·1 $\mu$g ml$^{-1}$) was prepared of each PAH to be identified and quantified. A 100-$\mu$l Hamilton microlitre syringe was fitted into a 'Linomat III' TLC plating machine (CAMAG, Switzerland). Plating was carried out at a band setting of 15 mm and a volume of 30 $\mu$l min$^{-1}$ in the sequence of standard, sample, standard, dropping 40 $\mu$l of the sample and 20 ml of the standard solution. Separation of the PAH from other compounds was achieved by inserting the TLC plate into the separation solution upright for 2 h. The separation solution contained 130 ml methanol, 20 ml ethyl acetate, 10 ml 2-methylpropan-1-ol, 10 ml toluene and 35 ml distilled water. Identification and quantitative determination was carried out against the standard in a spectrophotofluorimeter fitted with a graphic recorder at excitation and emission wavelengths set according to a table prepared by Tóth (1970). For example, for benzo(*a*)pyrene the wavelength settings were 385 nm for excitation and 430 nm for emission.

A recovery of 90–100% was achieved by this method as measured by the recovery of 10 $\mu$g benzo(*a*)pyrene added to the control meat samples.

The areas under the curves obtained were proportional to the amount of PAH. The areas for standards and samples were compared and the actual amounts of PAH in each sample were calculated and recorded. Ten replicate determinations were carried out on each sample.

## 3 RESULTS

Table 1 shows the range of PAH determined in the smoked meat samples and

**TABLE 1**
Polycyclic Aromatic Hydrocarbons Determined in Nigerian Smoked Meats, and their Carcinogenicity Ratings

| *No.* | *PAH* | *Amount* $\mu g\ kg^{-1}$ | *Carcino-genicity (USEPA 1975)* |
|---|---|---|---|
| 1 | Benzo(*a*)pyrene | 10·5–66·9 | +++ |
| 2 | Benzo(*c*)phenanthrene | 0·80–11·6 | +++ |
| 3 | Benzo(*b*)fluoranthene | 3·40–21·7 | ++ |
| 4 | Benzo(*j*)fluoranthene | 5·3–8·20 | ++ |
| 5 | Benzo(*a*)anthracene | 1·1–14·7 | + |
| 6 | Chrysene | 2·2–30·8 | + |
| 7 | Indeno(*cd*)pyrene | 1·4–5·50 | + |
| 8 | Benzo(*e*)pyrene | 1·9–10·0 | + |
| 9 | Cyclopenta(*cd*)pyrene | 0·4–5·50 | − |
| 10 | Benzo(*k*)fluoranthene | 1·9–10·3 | − |
| 11 | Benzo(*ghi*)perylene | 1·9–5·20 | − |
| 12 | Triphenylene | 0·5–1·10 | − |

+++=Highly carcinogenic; ++=moderately carcinogenic; += slightly carcinogenic; −=no evidence of carcinogenicity.

their carcinogenicity ratings (US-EPA 1975). From the ten replicate determinations carried out on each sample, benzo(*a*)pyrene has a range of 10·5–66·9 ng $g^{-1}$ with a mean of 41·0 ng $g^{-1}$. The fire-glow temperatures and the oven smoking temperatures for the 10 batches of meat samples were recorded. Glow temperatures ranged between 624 and 1160°C, mean 926°C; smoking temperatures ranged between 180 and 200°C, mean 191·5°C.

## 4 DISCUSSION

The method gave a recovery of 90–100% with spiked control samples. The usually hard meat samples were treated with liquid nitrogen to enable them to be finely minced, and were soaked in boiling water to promote better PAH recovery.

In Nigeria, these meats are smoked in open fireplaces or in drum or mud ovens using smoke from scrap wood. The origin of the PAH in kundi is the smoke produced from hard woods pyrolysed at high glow temperatures of 624–1160°C. The smoking temperatures reach 200°C. The critical aspect is that the meats are resmoked in the fire places until required, and may be resmoked for weeks to months. Meat retailers also resmoke unsold meats daily until disposed of. This practice of resmoking helps the build-up of benzo(*a*)pyrene and other PAH. Benzo(*a*)pyrene is a highly carcinogenic PAH and its presence in foods makes the food unfit for human consumption. Eleven other PAH, eight of them carcinogenic to various degrees, were determined.

The evidence provided here points to the fact that the polycyclic aromatic hydrocarbons are common and constitute major carcinogens in Nigeria. The detected benzo(*a*)pyrene levels of 10·5–66·9 $\mu g\ kg^{-1}$ are very high viewed against

the fact that a maximum of 1 $\mu$g kg$^{-1}$ is permitted in meats in Austria, Israel, Switzerland and West Germany and in meats exported to the USA (USEPA 1975; Walker 1977).

The epidemiological studies of Bailey and Dungal (1958) in Iceland correlating the high incidence of stomach cancer with the intake of home-smoked meat products is of interest in relation to the situation in Nigeria. Records in Ibadan and Kampala Medical Schools (Bababunmi *et al* 1978; Emerole 1980) have indicated that the incidence of primary liver and stomach cancers in Nigeria is very high compared with that in Europe and the USA. The consumption of smoked plant and animal products, among other factors such as hepatitis B virus and mycotoxins, contributes to this high incidence.

The kundi smoke-dried meat, as presently produced, creates high health risks for those consuming it. This calls for improved methods for smoking and preservation.

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# Use of Response Surface Methodology in the Development of Guava Concentrate

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*ABSTRACT*

*A response surface methodology (RSM) was used for the determination of optimum pH, processing temperature and °Brix to produce an acceptable guava concentrate. These three factors were used as independent variables whose effects on colour and viscosity of the concentrate, as well as on titratable acidity, body (mouthfeel), flavour and overall acceptability of the reconstituted juice were evaluated. The* $R^2$ *values for colour, titratable acidity and overall acceptability were greater than 0·9. Analysis of the regression coefficients showed that the pH of the concentrate was the most important factor affecting the characteristics of the product as it exerted a highly significant influence on the colour of the concentrate as well as on titratable acidity, flavour and overall acceptability of the juice. The colour, titratable acidity and overall acceptability were affected by °Brix whereas temperature influenced only the colour of the product. The combinations required to achieve suitable colour were pH 2·4–2·9, 75–81°C and 48–55°Brix, or pH 4·0, 87–95°C and 46°Brix. For acceptable flavour, pH 3·3–3·6, 78–86°C and 50·9°Brix are required, and for maximum overall acceptability, pH 3·3–3·9, 79·1°C and 35–55°Brix.*

*Key words*: Response surface methodology, guava concentrate, sensory quality.

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## 1 INTRODUCTION

Guava fruits (*Psidium guajava* L) of the Vietnamese type are now popular in Malaysia. Owing to the relative abundance of the fruit, there is currently a growing interest among local food manufacturers in finding ways to utilise the fruits other than for fresh consumption. The results of preliminary work have shown that this type has potential for juice preparation and canning in syrup (Yusof 1985).

Work on guava purées started many years ago (Ruehle 1948). Luh and co-workers (Luh 1971; Luh *et al* 1975) have described some formulae for the production of frozen guava concentrate. There have also been reports on the quality of aseptically filled guava purée (Chan and Cavaletto 1982) and its storage performance in plastic pouches (Kalra and Revathi 1981), but those products were different from the heat-processed guava concentrate studied here. The differences include: (a) variety; (b) sugar/acid range of the product; and (c) the end use of the product, eg for dilution to the required level of soluble solids (°Brix) producing a ready-to-drink guava nectar. Since pH, processing temperature and °Brix are the key factors determining the quality of most fruit products, they were chosen as independent variables in the present development work. The response surface methodology (RSM) employed by Daley *et al* (1978) was used to determine the optimum conditions of pH, temperature and °Brix. The principles of the approach were explained by Henika (1972, 1982) and Giovanni (1983). It proved very suitable for product development and was also employed by Henselman *et al* (1974).

## 2 EXPERIMENTAL

### 2.1 Materials

Batches of 6 kg of guava fruits of the Vietnamese type were purchased from a farm in Selangor. Table sugar (sucrose) was purchased in the normal manner, and food-grade citric acid was obtained locally. The ethylene gas used for ripening the fruits was obtained from the Malaysian Oxygen Co Ltd, Petaling Jaya, Selangor. The programmable PMC Dataplate hotplate/stirrer was purchased from Translab (M) Sdn Bhd, Kuala Lumpur.

### 2.2 Methods

The fruits as harvested were light green in colour and were ripened under ethylene to a yellow–green stage. Through previous experience, fruits at this stage were already soft (fruit hardness which was measured using an Instron Model 1140 was around 5 kg $cm^{-2}$) so it is easy to process. They had highest water soluble pectin content, brix acid ratio, vitamin C content and optimum fruit flavour and aroma. They were then washed and cut into small pieces and macerated with an equal weight of water in a Waring blender for 2 min. The purée was passed through a 2-mm pore size screen (mesh no 8) to remove the seeds and homogenised to

improve its texture (Ruehle 1948; Luh 1971). When required the purée was then adjusted to selected soluble solids levels in the range 35–55°Brix and pH values in the range 2·4–4·0 (Table 1) by addition of sugar and citric acid. The prepared purées were then heated using the programmable PMC Dataplate hotplate/stirrer for 5 min at selected heating temperatures between 75 and 95°C. An Abbé refractometer was used for measuring °Brix and the 'Jenway' digital pH meter for sample pH.

### 2.3 Experimental design and evaluation

RSM was used in designing this experiment (Giovanni 1983; Henika 1972). The independent variables pH, temperature and °Brix were coded as $X_1$, $X_2$ and $X_3$, respectively. Five levels of each of the three factors were chosen. A value of −1·682 was assigned to the lowest level, 0 to the middle level and +1·682 to the highest level. Values between −1·682 and +1·682 for the three factors were determined by calculation. Altogether 17 combinations (including three replicates of the centre point) were chosen in random order according to a central composite rotatable design configuration for three factors (Cochran and Cox 1975). The actual values of the three independent variables together with the responses are shown in Table 1. The responses measured were colour and viscosity of the concentrate plus titratable acidity, flavour, body (mouthfeel) and overall acceptability of the diluted juice.

Colour was measured by Hunter Colorimeter Model D 25 and by sensory panellists who were presented with the purée samples packed in transparent glass

**TABLE 1**
Effect of pH, Temperature and °Brix Combinations on Seven Dependent Variables

| *Independent variables* | | | *Dependent variables* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *pH* | *Temp. °C* | *°Brix* | *Concentrate* | | *Juice* | | | | |
| | | | *Hunter L value* | *Viscosity (centipoise)* | *Titratable acidity (%)* | *Colour score* | *Flavour score* | *Body score* | *Overall acceptability score* |
| 2·72 | 79·1 | 39·1 | 43·6 | 656 | 1·50 | 4·0 | 5·4 | 5·2 | 3·2 |
| 3·68 | 79·1 | 39·1 | 43·9 | 748 | 0·25 | 4·0 | 7·4 | 6·8 | 7·0 |
| 2·72 | 90·0 | 39·1 | 42·1 | 646 | 1·57 | 2·2 | 5·0 | 5·0 | 3·0 |
| 3·68 | 90·9 | 39·1 | 43·1 | 887 | 0·57 | 6·0 | 6·0 | 6·2 | 5·8 |
| 2·72 | 79·1 | 50·9 | 39·2 | 1096 | 0·97 | 6·2 | 5·4 | 6·0 | 5·4 |
| 3·68 | 79·1 | 50·9 | 38·2 | 818 | 0·31 | 6·0 | 7·0 | 6·6 | 6·2 |
| 2·72 | 90·9 | 50·9 | 39·2 | 1966 | 0·91 | 3·4 | 5·0 | 6·0 | 5·2 |
| 3·68 | 90·9 | 50·9 | 36·5 | 949 | 0·31 | 6·4 | 6·0 | 6·4 | 5·4 |
| 2·40 | 85 | 45 | 38·5 | 642 | 2·27 | 5·2 | 4·4 | 5·0 | 2·0 |
| 4·00 | 85 | 45 | 39·7 | 824 | 0·29 | 6·2 | 6·4 | 5·2 | 5·2 |
| 3·20 | 75 | 45 | 41·8 | 709 | 0·91 | 5·4 | 6·2 | 6·0 | 6·8 |
| 3·20 | 95 | 45 | 40·1 | 941 | 0·82 | 3·2 | 7·2 | 6·2 | 6·8 |
| 3·20 | 85 | 35 | 47·3 | 729 | 0·64 | 4·4 | 7·6 | 6·0 | 6·4 |
| 3·20 | 85 | 55 | 38·4 | 888 | 0·55 | 6·0 | 6·8 | 5·6 | 6·8 |
| 3·20 | 85 | 45 | 42·8 | 754 | 0·86 | 5·6 | 7·0 | 5·2 | 6·8 |
| 3·20 | 85 | 45 | 42·6 | 764 | 0·86 | 5·8 | 7·2 | 7·0 | 7·4 |
| 3·20 | 85 | 45 | 43·0 | 723 | 0·86 | 5·4 | 6·4 | 6·4 | 5·4 |

bottles; all 17 samples were tested at one sitting. Viscosity was determined using the Brookfield Viscometer, spindle no 2, at a speed of 3600 Hz. Titratable acidity was determined by titrating 5 ml of juice against 0·1 M NaOH using phenolphthalein as indicator (Ranganna 1977). Sensory analysis of flavour was carried out by five trained panellists. The flavour, juice body and overall acceptability judgments were made on purées diluted to 12°Brix. Samples for evaluation were chosen at random and the evaluation was conducted in two sessions, one in the morning and another in the afternoon. To ensure that their tastebuds were refreshed the panellists were instructed to chew plain crackers between samples.

The panellists first categorised the sample as to whether they liked or disliked the products and later scored the samples for preference based on a hedonic scale from 1–9 (1: dislike most; 9: like most) (Larmond 1970).

The data were analysed using the statistical analysis system (SAS) program package for analysis of variance and regression coefficient calculation. The regression coefficients were then used to make statistical calculation to generate contour maps from the regression models.

## 3 RESULTS AND DISCUSSION

### 3.1 Statistical analysis

Table 2 summarises the results of analysis of variance for each of the dependent variables with their corresponding coefficients of determination ($R^2$) (Little and Hills 1978; Mendenhall 1975). The closer the value of $R^2$ is to unity the better the empirical model fits the actual data. The smaller the value of $R^2$ the less relevance the dependent variables in the model have to explanation of the behaviour variation. Only $R^2$ values greater than 0·900 were considered accurate enough for prediction purposes.

The $R^2$ value of 0·712 for viscosity indicated that a higher degree model was

**TABLE 2**
Analysis of Variance for Seven Dependent Variables

| *Dependent variables* | $R^2$ | *Mean square regression* | *Mean square error* | F | *Probability* |
|---|---|---|---|---|---|
| *Concentrate* | | | | | |
| Colour (Hunter *L* value) | 0·980 | 13·118 | 0·344 | 38·19 | 0·000 1** |
| Colour (panellists) | 0·923 | 2·583 | 0·278 | 9·30 | 0·0038** |
| Viscosity | 0·712 | 12 055·91 | 62 690·82 | 1·92 | 0·200 4 |
| *Juice* | | | | | |
| Titratable acidity | 0·953 | 0·471 | 0·0300 | 15·69 | 0·000 7** |
| Juice body | 0·492 | 0·350 | 0·464 | 0·75 | 0·662 |
| Flavour | 0·797 | 1·288 | 0·422 | 3·05 | 0·078 |
| Overall acceptability | 0·910 | 3·837 | 0·482 | 7·96 | 0·006 1** |

**Highly significant; other figures not significant.

probably required to explain viscosity. Visual observations on the purée showed that there were variations in viscosity between different batches of samples. This difference might be due to variations in the stage of maturity of samples used; the difficulty in determining consistently the stage of maturity for guavas is well recognised (Yusof and Mohamed 1987). Some indices of maturity should be included in a new model if viscosity is to be explained further.

Theoretically, the viscosity of the purée is related to the body of the juice. In this experiment, since the final °Brix of samples was adjusted to 12, the juice body was influenced by the amount of purée sample taken for dilution and the quality of fruit pulp present in it. The very low $R^2$ (0·492) observed for the juice body indicated that this characteristic was not adequately described by pH, temperature and °Brix. Since the lack-of-fit test did not produce a significant $F$ value, it is likely that other independent variables need to be included in the model if juice body is to be adequately described. A value of 0·797 was observed for flavour, and the $F$ value was significant only at the 0·078 level. The model was considered approximate and used for trend analysis but not for prediction of optimum values. The $R^2$ values for Hunter colour, sensory colour, titratable acidity and overall acceptability were all greater than 0·90 (highly significant), and predictions concerning these responses could be made with confidence ($\alpha<0·01$).

Coefficient estimates for five regression models and the results of significant tests on the coefficients are shown in Table 3. From the significance tests on the estimates, pH was the most important factor influencing product quality. Its effect on titratable acidity, sensory colour, overall acceptability and flavour responses was highly significant. On the other hand its second order effect on overall acceptability and Hunter colour was highly significant but less so on titratable acidity and flavour. It was interesting to note that the effect of pH differed according to the method of measuring colour responses. The first order effect on

**TABLE 3**
Regression Coefficients for Five Dependent Variables

| *Coefficient* | *Colour (Hunter)* | *Titratable acidity* | *Colour sensory* | *Overall acceptability* | *Flavour* |
|---|---|---|---|---|---|
| $b_0$ (intercept) | 42·81** | 0·868** | 5·62** | 6·56** | 6·90** |
| $b_1$ | −0·03 | −0·50** | 0·61** | 0·95** | 0·66** |
| $b_2$ | −0·50* | 0·013 | −0·43* | −0·18 | −0·11 |
| $b_3$ | −2·53* | −0·113** | 0·62** | 0·28 | −0·13 |
| $b_1^2$ | −1·34** | 0·122** | 0·02 | −1·14** | −0·63* |
| $b_2^2$ | −0·68** | −0·024 | −0·52* | −0·009 | −0·17 |
| $b_3^2$ | −0·01 | −0·120 | −0·20 | −0·079 | −0·01 |
| $b_{12}$ | −0·13 | 0·039 | 0·875** | −0·20 | −0·20 |
| $b_{13}$ | −0·63* | 0·124 | −0·125 | −0·70* | −0·05 |
| $b_{23}$ | 0·08 | 0·056 | −0·325 | 0·05 | 0·05 |

Subscripts: 1=pH; 2=temperature; 3=°Brix.
*Significant at 0·05 level.
**Significant at 0·01 level.

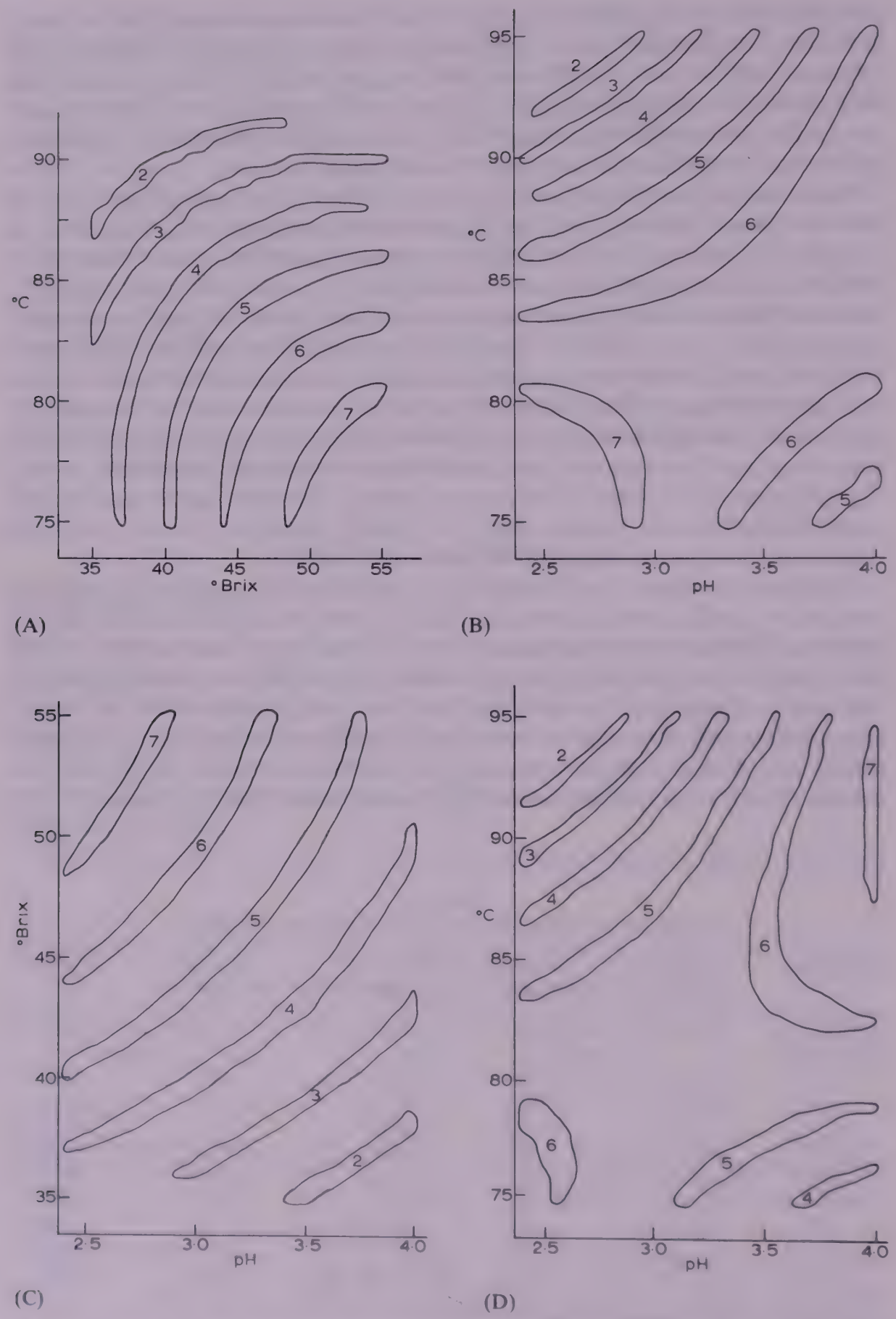
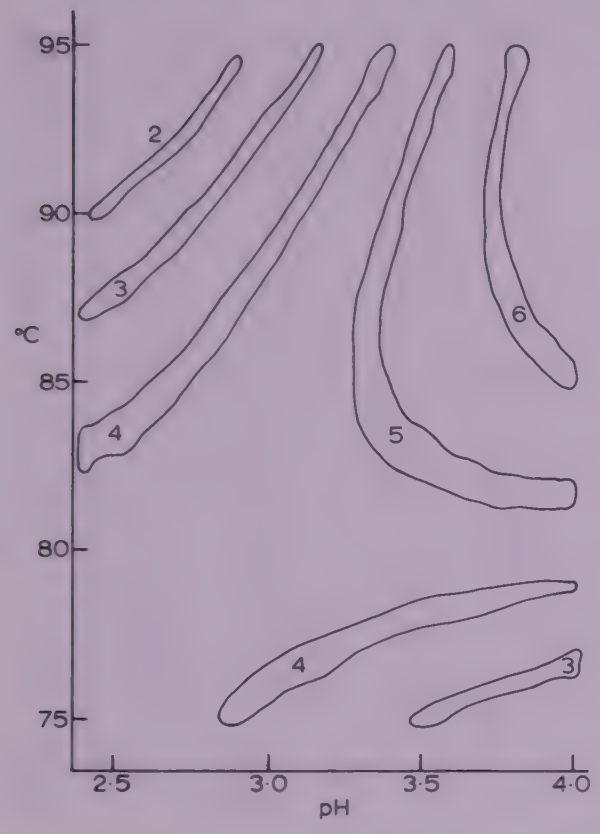
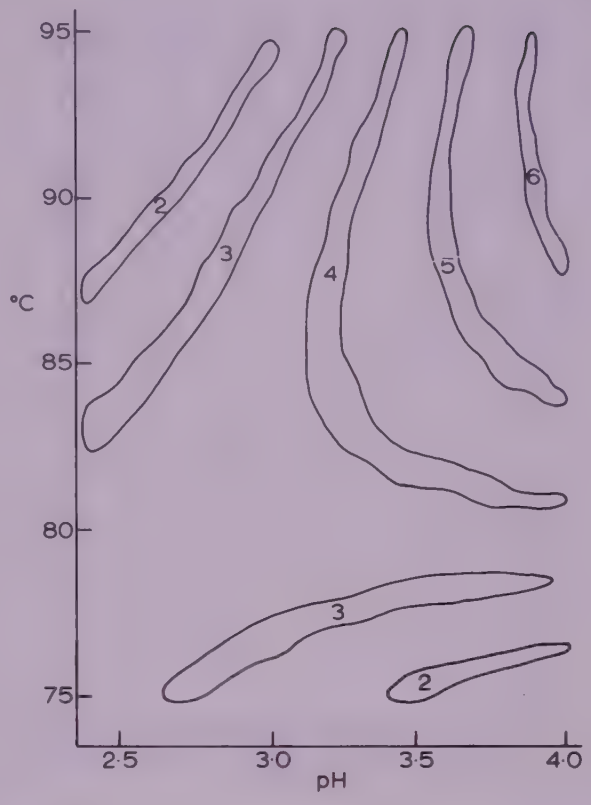


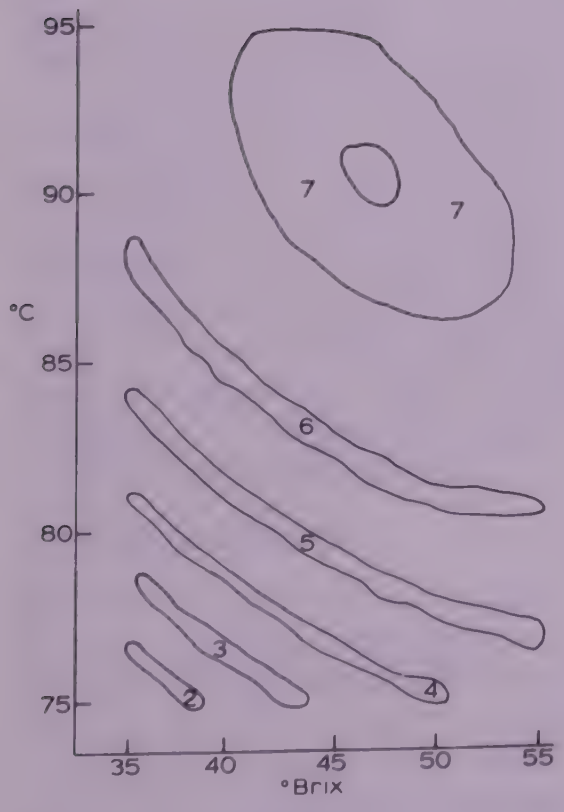

**Fig 1.** Contours for colour response. (A) Soluble solids vs temperature at pH 2·4. (B) pH vs temperature at 55·0°Brix. (C) pH vs soluble solids at 75·0°C. (D) pH vs temperature at 45·0°Brix.




(A)




(B)




(C)

**Fig 2.** Contours for colour response. (A) pH vs temperature at 39·0°Brix. (B) pH vs temperature at 35·0°Brix. (C) Brix vs temperature at pH 4·0.

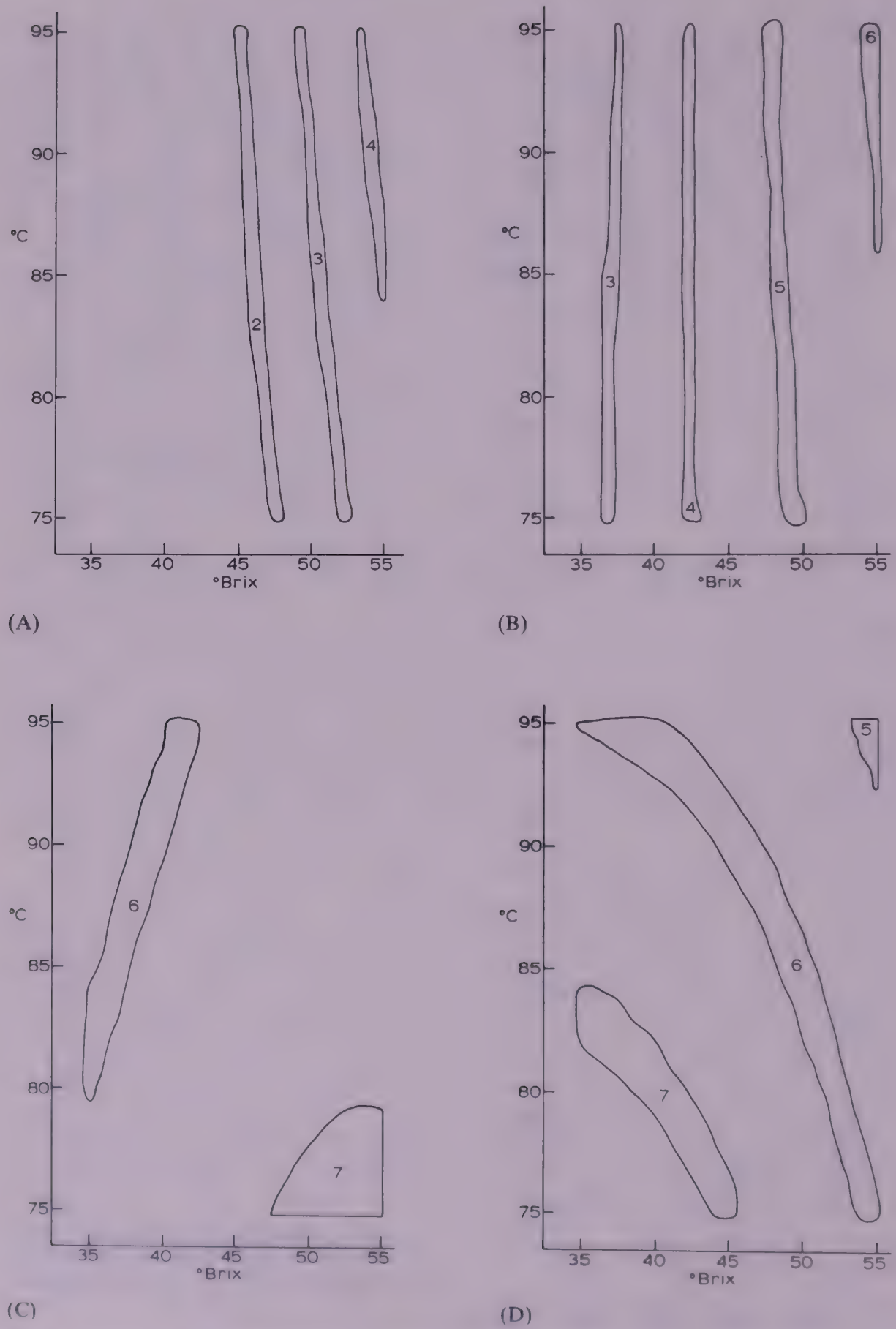
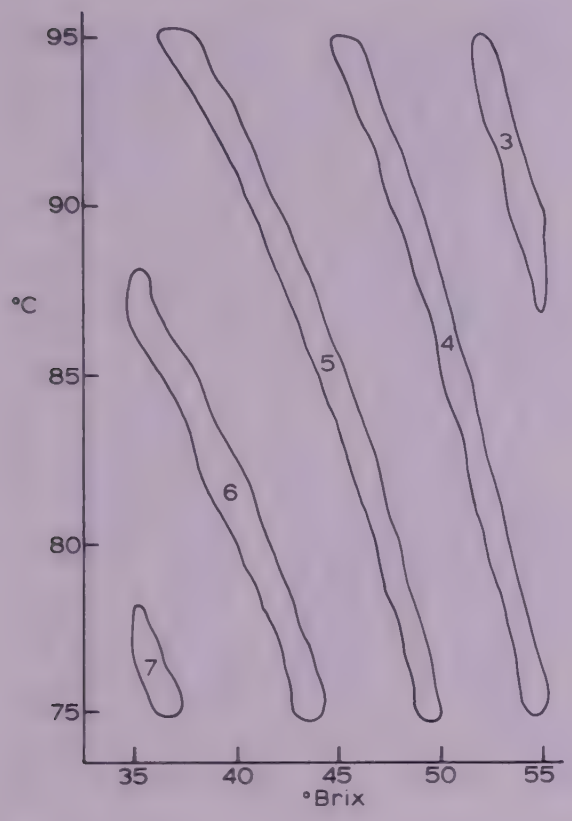



**Fig 3.** Contours for overall acceptability according to soluble solids and temperature at various pH. (A) At pH 2·4. (B) At pH 2·7. (C) At pH 3·2. (D) At pH 3·6.




(E)

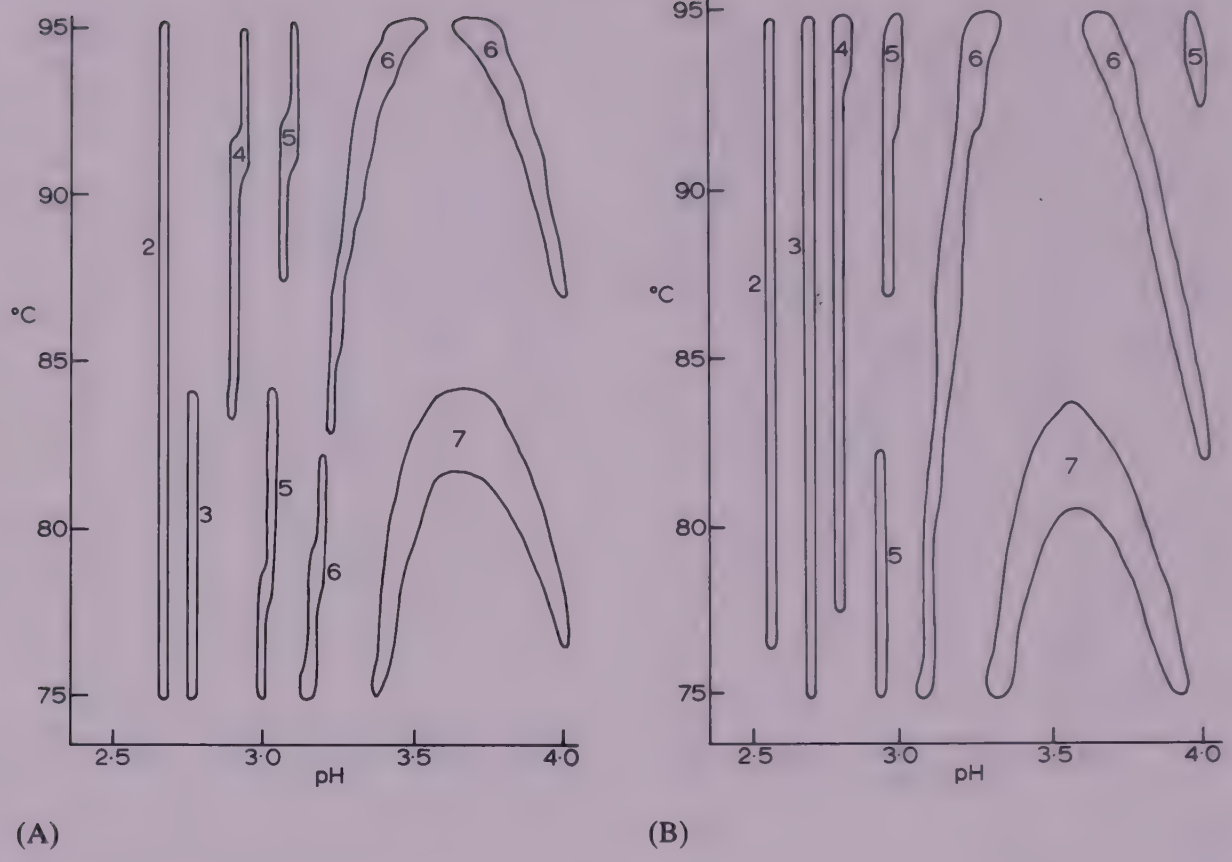
**Fig 3** — *contd.* (E) At pH 4·0.

colour was highly significant when assessed by panellists, but there was no significant effect when measured instrumentally. The reverse situation occurred with its second order effect. This interesting contrast between sensory and instrumental data was also observed by others (Daley *et al* 1978).

The next important factor was °Brix which had a major influence on the product. Its first order effect on colour was highly significant, as observed both by instrumental measurement and by sensory evaluation. It also showed a significant influence on titratable acidity. The interaction between °Brix and pH was significantly related to instrumental colour and overall acceptability. Temperature seemed to exert an influence only on the colour of the product in its first order effect, in its second order effect and when it interacted with pH. Among all responses measured, colour seemed to be most affected by the three factors examined (pH, temperature and °Brix), as indicated by both instrumental and sensory methods of assessment.

### 3.2 Response contours

In order to describe how the test variables affect the responses, contours of constant response values were computed for two significant responses (sensory colour and overall acceptability) and for flavour, for further analysis. Emphasis is placed on these three responses since the consumer is the ultimate judge and these characteristics need to be assessed in relation to the acceptability of the product to the consumer. However, as is the common limitation with all sensory data, results of this study cannot be taken to represent the preferences of consumers at large,



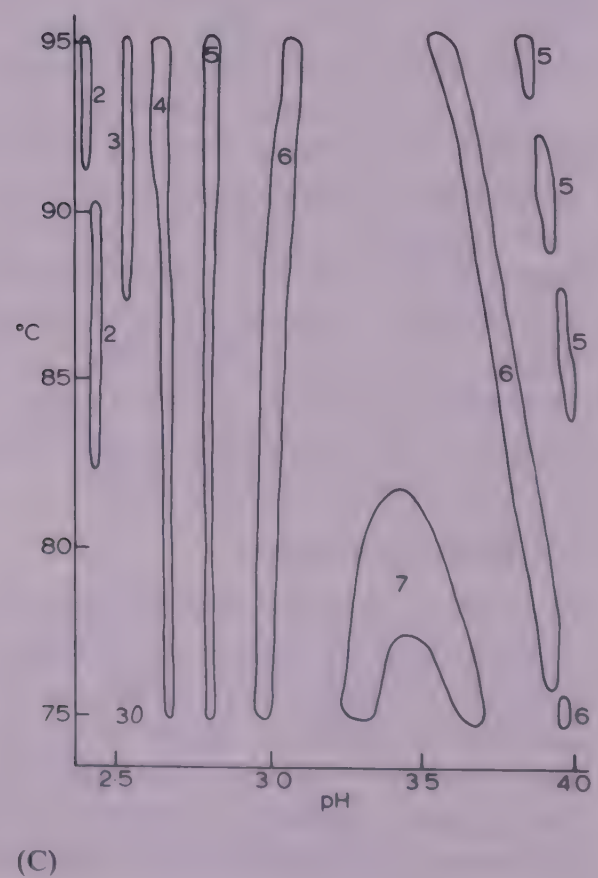
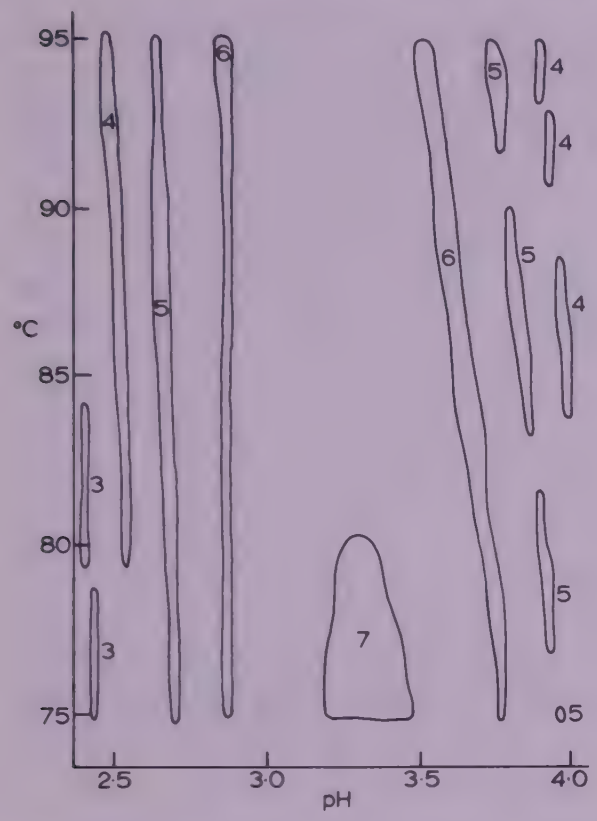
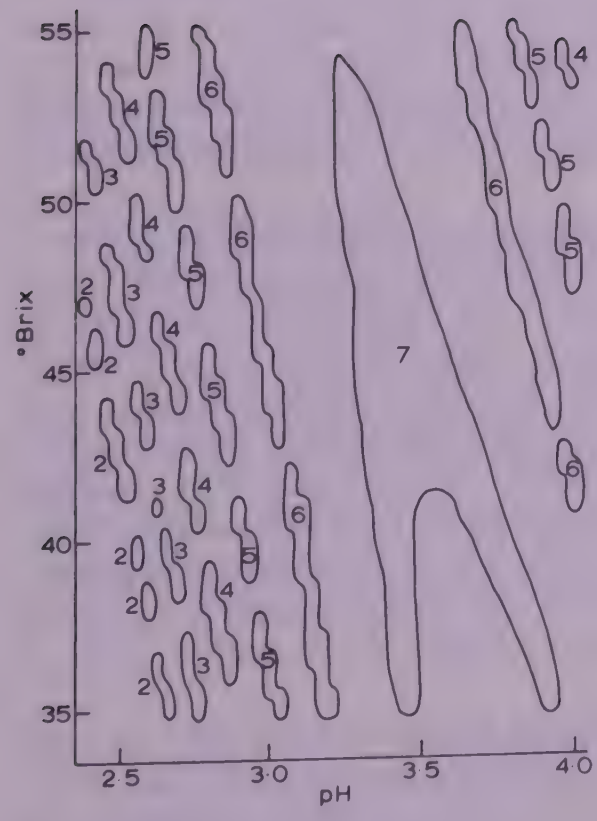




**Fig 4.** Contours for overall acceptability according to pH and temperature at various soluble solids levels. (A) At 35°Brix. (B) At 39·0°Brix. (C) At 45·0°Brix.




(D)


(E)




(F)

**Fig 4** — *contd.* (D) At 50·9°Brix. (E) At 55·0°Brix. (F) Contours for overall acceptability according to pH and soluble solids at 79·0°C.


**Fig 5.** Contours for optimum flavour response according to pH and temperature at 50·9°Brix.

even though panellists in this work provided reproducible results. The numbers that form the contour maps (Figs 1–5) are computer codes assigned to appropriate ranges of average panellists scores as follows: code 2 for scores greater than 1·9 and less than 2·1; 3 for scores greater than 2·9 and less than 3·1; 4 for scores greater than 3·9 and less than 4·1; 5 for scores greater than 4·9 and less than 5·1; 6 for scores greater than 5·9 and less than 6·1; 7 for scores greater than 6·9 and less than 7·1. Such codings were used for each of the three types of response covered in this paper.

Figures 1A, B and C show the contour maps for the response by panellists to colour. The contours indicate that when the product was prepared at low temperature (75–81°C) it required low pH (2·4–2·9) and high solids (48–55°Brix) for the preferred colour to develop. Decreasing the solids to 45°Brix required higher temperature (87–94·5°C) and higher pH (4·0) to produce the desired colour (Fig 1D). When the solids were reduced further to 39·1 and 35°Brix, product colour was given low preference values (Figs 2A and B). The optimum °Brix value for desired colour was 46 (range 40–54°Brix), at which level the product to be adjusted to pH 4·0 and processed at 86·5–95°C (Fig 2C). The Hunter *L* values for the entire series of samples were between 36·5 and 476·3 (Table 1). It was found that the panellists preferred a product coloured slightly brown, with *L* values of 38–42.

The contour maps for overall acceptability at a series of fixed pH values are given in Fig 3. At very low pH (2·4 and 2·7; Figs 3A and B) acceptance of the product was low. At moderately low pH (3·2; Fig 3C) high preference was indicated for products having Brix values in the range 47–55°Brix. However, when the pH was increased to 3·68 (Fig 3D) and 4·0 (Fig 3E), the contours turned

in the opposite direction towards the origin, approaching it at pH 4·0. Thus, as the pH increased, less in the way of solids was required to increase acceptability, ie the less sour the product, the less the amount of sugar needed to make it organoleptically desirable. This serves to reinforce the importance of the already well established relationship between degrees of acidity and sweetness and the widely used term 'Brix/acid ratio'. The relationship between acidity and °Brix can be seen more clearly in the series of Fig 4, where °Brix was kept constant. At low values (35°Brix; Fig 4A) the acceptable pH range for samples was relatively broad, pH 3·4–4·0. As solids levels were increased to 39, 45 and 50·9°Brix (Figs 4B, 4C, 4D) the pH range shifted downwards and became narrower (pH 3·3–3·9, 3·2–3·7, 3·2–3·5, respectively). Finally, at 55°Brix (Fig 4E), the acceptable range was reduced to ~pH 3.1–3.3. The shifting of the curve with varying solids clearly indicated that the amount of acid present should be just sufficient to give the right tartness to the product. The situation is best illustrated as in Fig 4F, where temperature was kept constant at 79·1°C. High scores were obtained for samples having solids in the range 35–55°Brix and pH in the range 3·3–3·9. The Brix/acid ratios of preferred samples were between 13 and 22. The titratable acidity value within the acceptable pH range was 0·3–0·9%. The low temperature range 75–84·5°C favoured high acceptability.

The results indicated that pH strongly influenced flavour since low responses were obtained at pH 2·4–2·7. Low temperatures (below 90°C) and medium to high pH (3·2–4·0) were required to bring out the guava flavour; an optimum guava flavour was developed at pH 3·3–3·6; 78–86°C and 50·9° Brix (Fig 5).

## 4 CONCLUSION

There are several possible combinations of pH, temperature and °Brix for producing good-quality guava concentrates:

(a) for acceptable colour
  (i) pH 2·4–2·9, 75–81°C and 48–55°Brix
  (ii) pH 4·0, 87–95°C and 46°Brix
(b) for acceptable flavour
  pH 3·3–3·6, 78–86°C and 50·9°Brix
(c) for maximum overall acceptability
  pH 3·3–3·9, 79·1°C and 35–55°Brix.

The choice will depend on the emphasis required by the processor. A particular combination could be chosen on the basis of consumer preference, economics, product shelflife, or some other consideration, or suitable combinations of these.

## ACKNOWLEDGEMENTS

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# Nutrient Content of Milk and Milk Products: Vitamins of the B Complex and Vitamin C in Retail Cheeses

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*ABSTRACT*

*A survey was undertaken to update and extend information on the water-soluble vitamin content of 42 different varieties of cheese on retail sale in the UK.*

*Key words*: Cheeses, vitamin levels, microbial synthesis.

## 1 INTRODUCTION

Differences in cheese-making procedures and the subsequent ripening period might well be expected to influence the levels of vitamins in the finished cheese. The water-soluble nature of the B vitamins and vitamin C means that a variable percentage of the vitamins contained in the starting milk is lost in the whey. The concentration of the vitamins in the cheese may also be subjected to changes during the ripening period due to their utilisation and synthesis by microorganisms.

This study was undertaken to assess the levels of water-soluble vitamins in a range of different types of cheese on retail sale.

## 2 EXPERIMENTAL

### 2.1 Samples (see Table 1)

Wherever possible at least 10 samples of each cheese variety, which included wrapped and/or prepackaged cheese, were obtained from various retail outlets in

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the Reading area during 1984/85. All individual samples of each cheese variety were bulked to give a single representative sample.

### 2.2 Handling and preparation of samples

All samples were placed in cool boxes (~5°C) immediately after purchase, transported to the laboratory and held in a refrigerator. With the exception of the soft cheeses referred to below, all samples were removed from the refrigerator on the day of purchase for immediate preparation in the following manner. Each individual sample of a particular cheese variety weighing ~225 g had any inedible part of the cheese (rind, skin, etc) removed (edible mould coats were not removed) and was then cut into six pieces. These pieces were grated using a Moulinex Charlotte 3 fitted with shredder attachment. The grated cheese was mixed thoroughly and 200 g aliquots were vacuum sealed in 125 $\mu$m gauge polythene bags for storage at −20°C. Cottage cheese, cheese spreads, quark and cream cheese were creamed and mixed, then packed and stored as above. All samples were kept chilled prior to grating and packing to diminish losses of fat and moisture.

### 2.3 Preparation of samples prior to analysis

Each frozen pack of cheese was brought to room temperature in the dark, regrated and homogenised under nitrogen with three-quarters of its weight of distilled water that had been boiled and cooled to ~40°C. Vitamin C was analysed on a ~25-g portion of the homogenate immediately after homogenisation. For folic acid, 1 g of a solution of phosphate ascorbic acid buffer pH 7·8 (Ford and Scott 1968) was added to 2 g of the homogenate; this was stored at −20°C together with ~5-g portions of the homogenate for later analysis of the other B vitamins. The vacuum-packed samples of cottage cheese, cheese spreads, quark and cream cheese were broken into pieces and grated while still frozen and then treated in the same way as the other samples.

### 2.4 Analytical methods

Vitamin C was measured essentially as described by Deutsch and Weeks (1965). Microbiological analyses of thiamin, riboflavin, vitamin $B_6$, nicotinic acid, folic acid, vitamin $B_{12}$, pantothenic acid and biotin were carried out as described by Scott *et al* (1984), but pantothenic acid was analysed after extraction with chicken liver enzyme and alkaline phosphatase (EC 3.1.3.1) and biotin after extraction with 2 M $H_2SO_4$.

## 3 RESULTS AND DISCUSSION

The majority of cheeses are produced from milk curd so the greater part of the water-soluble vitamins are lost in the whey. Even with vitamins present in a protein-bound form in milk, especially folic acid and $B_{12}$, a considerable proportion may be released during processing. However, there may also be an increase arising from microbial biosynthesis during ripening and storage, depending to

some extent on the starter used. Some mould-coated cheeses contain a higher concentration of certain vitamins than other cheeses.

Paul and Southgate (1978) state '... the values for the B vitamin in cheeses show great variability. This is due to the synthesis of these vitamins by the microorganisms involved in cheese production. The values may therefore vary according to the stage of maturity of the cheese and, particularly for Brie and Camembert type of soft cheeses, with the proportion of rind incorporated into the sample, because the concentration of many B vitamins is very much higher in the rind than in the body of the cheese.'

The present study was not designed to investigate such areas as changes during the ripening period or variations in concentration of vitamins in different parts of the cheese, but to provide a detailed analysis of the vitamins and other nutrients in a wide range of cheese representative of the current retail market in the UK, ie as sold to the consumer. In addition, the sampling protocol, designed to give a single representative sample of each variety, does not allow for any within-variety comparison.

It is very difficult to compare these results (see Table 1) directly with published tables of composition because of differences in data presentation, ie groupings of cheese varieties and averaging of data covering a wide spread of individual figures. However, with one or two exceptions, discussed later, the results of this study are in general agreement with previously published data.

It can be seen from Table 1 that the vitamin contents of hard and semi-hard cheese are very similar, but the contents of certain vitamins in mould-coated cheeses, particularly vitamin $B_6$, nicotinic acid, biotin and folid acid, and blue cheese, particularly vitamin $B_6$, nicotinic acid, pantothenic acid and folic acid, are higher than in other cheeses. Overall levels in cream cheese tended to be lower than those in other cheeses. To enable a very general comparison to be made, Table 2 gives the results from this study alongside those in groupings of cheeses given by Paul and Southgate (1978) and the means of data from various other sources.

A varying proportion of the water-soluble B vitamins may be lost from the milk depending on the severity of heat treatment; however, the greatest part is lost into the whey. Losses of thiamin, nicotinic acid and folic acid are 80–90%, of riboflavin and biotin 70–80%, vitamin $B_6$ and pantothenic acid 55–75% and vitamin $B_{12}$ 40–70% (Renner 1983).

The highest concentrations of vitamin $B_6$ (2·18 $\mu g\ g^{-1}$), nicotinic acid, (9·64 $\mu g\ g^{-1}$), biotin (0·076 $\mu g\ g^{-1}$) and folic acid (1·02 $\mu g\ g^{-1}$) were found in Camembert cheese; the highest level of thiamin (0·5 and 1·51 $\mu g\ g^{-1}$) in cheese spread; of riboflavin (6·51 $\mu g\ g^{-1}$) in Roquefort; of pantothenic acid (5·36 $\mu g\ g^{-1}$) in Blue Stilton; and of vitamin $B_{12}$ (0·021 $\mu g\ g^{-1}$) in Edam and Mozzarella. The lowest level of thiamin (0·24 $\mu g\ g^{-1}$) was in Cheddar; of riboflavin (1·58 $\mu g\ g^{-1}$) and vitamin $B_6$ (0·54 $\mu g\ g^{-1}$) in cream cheese; of nicotinic acid (0·4 $\mu g\ g^{-1}$) in Gruyère; of biotin (0·014 $\mu g\ g^{-1}$) in Gouda; of folic acid (0·12 $\mu g\ g^{-1}$) in Parmesan and Gruyère; and of vitamin $B_{12}$ (0·0039 $\mu g\ g^{-1}$) in Roquefort.

Differences between data from this survey and most published data are apparent for nicotinic acid in Brie, and pantothenic in Brie and Camambert which

**TABLE 1**
Content of Water-soluble Vitamins in Cheeses ($\mu g\ g^{-1}$)

| *Cheese type* | *Variety* | *No of samples* | *No of outlets from which samples obtained* | *Total vitamin C* | *Thiamin*[a] | *Ribo-flavin* | *Vitamin $B_6$*[a] | *Vitamin $B_{12}$* | *Nico-tinic acid* | *Panto-thenic acid*[a] | *Biotin* | *Folic acid* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Very hard | Parmesan | 17 | 6 | 0·1 | 0·34 | 4·37 | 1.31 | 0·019 | 1·16 | 4.26 | 0·033 | 0·12 |
| Hard (Ls Ns) | Edam | 10 | 5 | 1·8 | 0·29 | 3.45 | 0·86 | 0·021 | 0·70 | 3·79 | 0·018 | 0·40 |
| | Gouda | 10 | 7 | 1·3 | 0·27 | 3·03 | 0·76 | 0·017 | 0·55 | 3·21 | 0·014 | 0·43 |
| | Cheshire | 10 | 5 | 0·9 | 0·33 | 4·77 | 0·87 | 0·009 | 1·09 | 3·10 | 0·040 | 0·40 |
| Hard (Ms Ns) | English Cheddar | 10 | 5 | 1·4 | 0·34 | 4·16 | 1·01 | 0·012 | 0·89 | 3·81 | 0·031 | 0·37 |
| | Cheddars[b] | 47 | 3–5 | 2·4 | 0·24 | 4·45 | 0·99 | 0·010 | 0·46 | 3·35 | 0·028 | 0·30 |
| | Leicester | 10 | 5 | 1·2 | 0·32 | 4·59 | 1·08 | 0·012 | 0·87 | 3·78 | 0·030 | 0·24 |
| | Double Gloucester | 10 | 5 | 0·8 | 0·29 | 4·46 | 1·06 | 0·013 | 0·71 | 3·21 | 0·031 | 0·30 |
| | Derby | 10 | 1 | 0·3 | 0·27 | 4·10 | 0·98 | 0·014 | 0·33 | 2·91 | 0·030 | 0·26 |
| | Sage Derby | 7 | 4 | 0·6 | 0·31 | 4·34 | 0·94 | 0·014 | 0·54 | 3·36 | 0·032 | 0·24 |
| | Red Windsor | 10 | 6 | 1·0 | 0·34 | 3·71 | 1·02 | 0·014 | 1·03 | 4·06 | 0·026 | 0·32 |
| Hard (Hs Pr) | Gruyère | 10 | 7 | 0·6 | 0·30 | 3·89 | 1·13 | 0·016 | 0·40 | 3·51 | 0·015 | 0·12 |
| Semi-hard (Ns) | Lancashire | 10 | 4 | 1·5 | 0·32 | 4·53 | 0·84 | 0·011 | 1·08 | 2·75 | 0·040 | 0·44 |
| | Wensleydale | 10 | 5 | 1·1 | 0·30 | 4·63 | 0·91 | 0·011 | 1·08 | 3·00 | 0·040 | 0·43 |
| | Caerphilly | 10 | 4 | 3·5 | 0·31 | 4·72 | 1·04 | 0·011 | 1·09 | 2·92 | 0·035 | 0·50 |
| | White Stilton | 8 | 5 | 1·6 | 0·27 | 3·68 | 0·75 | 0·013 | 0·94 | 2·28 | 0·029 | 0·52 |
| Soft (unripened) | Cottage low fat | 10 | 6 | 2·0 | 0·35 | 2·63 | 0·76 | 0·007 | 1·35 | 3·97 | 0·030 | 0·27 |
| | Cottage flavoured | 10 | 4 | 6·1 | 0·56 | 2·09 | 0·82 | 0·006 | 1·94 | 3·14 | 0·030 | 0·13 |
| Soft (Ns) | Lactic cheese spread | 18 | 2 | 0·0 | 0·32 | 2·90 | 0·76 | 0·005 | 1·63 | 2·60 | 0·035 | 0·31 |
| | Quark | 9 | 6 | 6·5 | 0.37 | 3·02 | 0·79 | 0·007 | 1·93 | 4·36 | 0·030 | 0·45 |
| | Cream cheese | 17 | 4 | 0·9 | 0.33 | 1·58 | 0·54 | 0·003 | 0·68 | 3·22 | 0·019 | 0·13 |
| Soft (Sm or Hm) | Brie | 10 | 7 | 0·8 | 0·43 | 4·27 | 1·49 | 0·012 | 4·31 | 3·54 | 0·056 | 0·58 |
| | Camembert | 10 | 7 | 0·8 | 0·46 | 5·17 | 2·18 | 0.011 | 9·64 | 3·62 | 0·076 | 1·02 |
| Soft (acid) | Mozzarella | 10 | 5 | 2·8 | 0·30 | 3·11 | 0·90 | 0·021 | 0·76 | 2·52 | 0·022 | 0·19 |
| Processed | Processed | 14 | 4 | — | 0·28 | 2·81 | 0·78 | 0·009 | 1·02 | 3·08 | 0·023 | 0·18 |
| | Processed smoked | 13 | 6 | 0·2 | 0·26 | 2·65 | 0·75 | 0·009 | 0·61 | 2·24 | 0·015 | 0·18 |
| Processed soft | Cheese spread | 14 | 5 | 2·7 | 0·50 | 3·58 | 0·78 | 0·006 | 1·17 | 5·08 | 0·036 | 0·19 |
| | Cheese spread, flavoured | 31 | 4 | 0·5 | 1·51 | 2·50 | 0·77 | 0·006 | 1·58 | 3·07 | 0·024 | 0·19 |
| Blue, semi-hard | Blue Stilton | 10 | 4 | 0·4 | 0·27 | 4·33 | 1·61 | 0·010 | 4·91 | 7·10 | 0·036 | 0·77 |
| | Danish blue | 10 | 7 | 0·2 | 0·29 | 4·06 | 1·08 | 0·010 | 4·76 | 5·26 | 0·027 | 0·50 |
| Blue soft | Lymeswold | 10 | 7 | 0·6 | 0·36 | 4·25 | 1·42 | 0·009 | 5·61 | 3·92 | 0·063 | 0·56 |
| Sheep's milk—blue | Roquefort | 15 | 7 | 0·8 | 0·43 | 6·51 | 0·91 | 0·004 | 5·70 | 4·98 | 0·023 | 0·45 |
| Sheep's milk | Feta | 7 | 4 | 3·4 | 0·44 | 3·21 | 0·74 | 0·007 | 1·91 | 3·57 | 0·024 | 0·30 |
| Others | Tendale Cheddar | 10 | 5 | 1·1 | 0·30 | 5·30 | 1·25 | 0·013 | 0·93 | 5·15 | 0·038 | 0·56 |
| | Tendale Cheshire | 10 | 2 | 2·7 | 0·47 | 5·63 | 1·02 | 0·014 | 1·78 | 5·10 | 0·050 | 0·58 |
| | Vegetarian Cheddar | 8 | 4 | 1·1 | 0·34 | 4·51 | 1·10 | 0·012 | 0·44 | 4·57 | 0·026 | 0·25 |

[a]Expressed as thiamin HCl, pyridoxal HCl and Ca-D-pantothenate, respectively.
[b]Means for English, Canadian, Australian, Irish and New Zealand cheddar.
Ls: low scald; Ms: medium scald; Hs: high scald; Ns: normal starter; Pr: propionic eyehole; Sm or Hm: smear coat or surface mould.

**TABLE 2**
Comparison of Content of Water-soluble Vitamins in Cheeses with Published Data ($\mu g\ g^{-1}$)

| *Source* | *Varieties* | *Thiamin* | *Ribo-flavin* | *Vitamin $B_6$* | *Vitamin $B_{12}$* | *Nicotinic acid* | *Panto-thenic acid* | *Biotin* | *Folic acid* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Parmesan | 0·34 | 4·4 | 1·30 | 0·019 | 1·20 | 4·3 | 0·033 | 0·12 |
| 2 | Parmesan | 0·20 | 5·0 | 1·00 | 0·015 | 3·00 | 3·0 | 0·017 | 0·20 |
| 3 | Parmesan | 0·21 | 5·4 | 0·94 | — | 2·00 | 5·2 | 0·030 | 0·13 |
| 1 | Edam, Gouda | 0·28 | 3·3 | 0·81 | 0·019 | 0·63 | 3·5 | 0·016 | 0·42 |
| 2 | Edam, Gouda, St Paulin | 0·40 | 4·0 | 0·80 | 0·014 | 0·60 | 3·0 | 0·015 | 0·20 |
| 3 | Edam, Gouda | 0·47 | 3·3 | 0·70 | 0·018 | 0·75 | 3·3 | 0·015 | 0·23 |
| 1 | Cheddar, other hard cheese except Edam/Gouda | 0·30 | 4·4 | 0·99 | 0·013 | 0·68 | 3·4 | 0·028 | 0·27 |
| 2 | Cheddar, Cheshire, Gruyère, Emmental | 0·40 | 5·0 | 0·80 | 0·015 | 1·00 | 3·0 | 0·017 | 0·20 |
| 3 | Cheddar, Cheshire | 0·36 | 4·7 | 0·62 | 0·011 | 1·00 | 2·8 | 0·021 | 0·15 |
| 1 | White Stilton | 0·27 | 3·7 | 0·75 | 0·013 | 0·94 | 2·3 | 0·029 | 0·52 |
| 2 | White Stilton | 0·70 | 3·0 | — | — | — | — | — | — |
| 1 | Camembert, Brie | 0·45 | 4·8 | 1·90 | 0·012 | 7·00 | 3·6 | 0·066 | 0·80 |
| 2 | Camembert, Brie | 0·50 | 6·0 | 2·00 | 0·012 | 8·00 | 14·0 | 0·060 | 0·60 |
| 3 | Camembert, Brie | 0·49 | 5·5 | 2·50 | 0·015 | 8·80 | 7·1 | 0·056 | 0·64 |
| 1 | Cream | 0·33 | 1·6 | 0·54 | 0·003 | 0·68 | 3·2 | 0·019 | 0·13 |
| 2 | Cream | (0·20) | (1·4) | (0·10) | (0·003) | (0·80) | (—) | (—) | (0·05) |
| 1 | Blue Stilton, Danish Blue | 0·28 | 4·2 | 1·40 | 0·010 | 4·90 | 6·2 | 0·032 | 0·64 |
| 2 | Danish Blue, Roquefort | 0·30 | 6·0 | 1·50 | 0·012 | 9·00 | 20·0 | 0·015 | 0·50 |
| 3 | Blue | 0·36 | 5·0 | 1·40 | 0·012 | 5·90 | 14·8 | 0·033 | 0·45 |
| 1 | Processed | 0·28 | 2·8 | 0·78 | 0·009 | 1·00 | 3·1 | 0·023 | 0·18 |
| 1 | Soft (cheese spread) | 0·50 | 3·6 | 0·78 | 0·006 | 1·20 | 5·1 | 0·036 | 0·19 |
| 2 | Processed | 0·20 | 2·9 | — | — | 0·70 | — | — | 0·02 |
| 2 | Cheese spread | 0·20 | 2·4 | 0·60 | — | — | — | — | — |
| 3 | Processed | 0·31 | 4·1 | 0·71 | 0·005 | 1·30 | 5·3 | 0·033 | 0·26 |

Source: 1. This survey; 2. Paul and Southgate (1978); 3. Others: means of Renner (1983), Schertz and Kloos (1981) and Hartman and Dryden (1974).

fall at the bottom of the range given by Paul and Southgate (1978). Nicotinic acid values for Blue type are below the average given by Paul and Southgate (1978) but fall well within the given range. Pantothenic acid values are below the mean and fall outside the range.

Not only is Vitamin C water soluble, but it is also particularly labile; thus handling and subsequent processing mean that cheese has little or no vitamin C.

## ACKNOWLEDGEMENTS

This study forms part of a survey of the nutrient content of milk and milk products commissioned by the Food Science Division of the Ministry of Agriculture, Fisheries and Food, London. The contribution to the initiation and detailed planning of this study made by Dr D H Buss, Dr Hazel Tyler and Miss Helen Crawley is gratefully acknowledged.

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# Nutrient Content of Milk and Milk Products: Vitamins of the B Complex and Vitamin C in Retail Creams, Ice Creams and Milk Shakes

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*ABSTRACT*

*A study was undertaken to update and extend information on the water-soluble vitamin content of creams, ice creams and take-away milk shakes on retail sale in the UK. The concentration of water-soluble vitamins in creams reflected to varying extents the level of fat and the type of heat processing. The concentration of vitamins in both dairy and non-dairy ice creams were generally similar. The amounts of vitamins in milk shakes were similar to those in pasteurised milk.*

*Key words*: Creams, ice creams, milk shakes, B vitamins, vitamin C.

## 1 INTRODUCTION

Developments in processing technology, packaging and sales techniques have led to a widening of the choice of creams and ice creams available to the consumer but there are few analytical data available on their water-soluble vitamin content. This study was undertaken to assess the levels of water-soluble vitamins in a range of creams, dairy and non-dairy ice creams and 'take-away' milk shakes on retail sale.

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## 2 EXPERIMENTAL

### 2.1 Samples (see Table 1)

*2.1.1 Creams*

With the exception of certain of the frozen samples which were purchased in London, all samples (own and brand labels) were purchased during June 1984 from retail outlets in the Reading area.

Samples were transferred to a cool box (~5°C) immediately after purchase, transported to the laboratory and then refrigerated until they were prepared for analysis the day after purchase. Frozen samples were stored at −20°C prior to preparation for analysis.

*2.1.2 Ice creams*

All samples were purchased from retail outlets in the Reading area during June 1985. As soon as possible after purchase, samples were refrigerated or stored at −20°C prior to preparation for analysis.

*2.1.3 Milk shakes*

All samples were purchased from 'take-away' outlets in the Reading area during June 1985. Samples were stored in a refrigerator prior to preparation for analysis on the day of purchase.

### 2.2 Preparation of samples

*2.2.1 Creams*

Refrigerated samples (in their containers) were warmed in a water bath to 37°C then gently mixed. Equal quantities of each individual sample of the same type of cream (ie single, double, etc) were bulked and gently mixed to give a single sample representative of that type. Frozen samples were removed from their retail bags, transferred to a 5-litre glass vessel and thawed in a water bath at 37°C prior to mixing. Vitamin C was analysed immediately on ~25 g of the bulked sample. For folic acid, 1 g of a solution of phosphate ascorbic acid buffer pH 7·8 was added to 2 g of sample and stored at −20°C together with ~5-g portions of the sample for later analysis of the other B vitamins.

*2.2.2 Ice creams*

Equal quantities of each sample of the same type of ice cream (eg dairy or non-dairy) were transferred to a covered container which was placed initially in an incubator and then in a water bath both at 37°C. After thawing, samples containing particulate matter, such as pieces of fruit, chocolate and nuts, were homogenised gently under nitrogen. Samples were then gently mixed to give a single sample representative of ice cream type. Samples for analysis were taken as described above for creams.

*2.2.3 Milk shakes*

Equal quantities of each individual sample were bulked and gently mixed to give one representative sample. Samples for analysis were taken as described above for creams.

**TABLE 1**
Content of Water-soluble Vitamins in Creams ($\mu g\ g^{-1}$)

| *Samples* | *No of samples* | *No of outlets from which samples obtained* | *Total vitamin C* | *Thiamin*[a] | *Riboflavin* | *Vitamin* $B_6$[a] | *Vitamin* $B_{12}$ | *Nicotinic acid* | *Panto-thenic acid*[a] | *Biotin* | *Folic acid* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Fresh* | | | | | | | | | | | |
| Half cream | 10 | 5 | 11·7 | 0·33 | 1·77 | 0·48 | 0·0025 | 0·65 | 2·57 | 0·017 | 0·064 |
| Single cream | 10 | 5 | 8·9 | 0·36 | 1·67 | 0·48 | 0·0025 | 0·70 | 2·84 | 0·018 | 0·068 |
| Whipping cream | 10 | 5 | 7·4 | 0·24 | 1·65 | 0·37 | 0·0021 | 0·39 | 2·21 | 0·014 | 0·070 |
| Double cream | 12 | 5 | 6·1 | 0·22 | 1·57 | 0·28 | 0·0019 | 0·39 | 1·91 | 0·011 | 0·066 |
| Clotted cream | 17 | 7 | 0·4 | 0·16 | 1·62 | 0·25 | 0·0014 | 0·44 | 1·36 | 0·010 | 0·060 |
| *UHT* | | | | | | | | | | | |
| Half cream | 5 | 1 | 0·4 | 0·31 | 1·69 | 0·41 | 0·0018 | 0·79 | 2·82 | 0·018 | 0·027 |
| Single cream | 8 | 4 | 0·2 | 0·29 | 1·66 | 0·39 | 0·0022 | 0·68 | 2·70 | 0·019 | 0·027 |
| Whipping cream | 10 | 5 | 0·2 | 0·26 | 1·64 | 0·32 | 0·0017 | 0·50 | 2·27 | 0·014 | 0·015 |
| Spray cream | 8 | 3 | 0·0 | 0·25 | 1·68 | 0·32 | 0·0021 | 0·49 | 1·95 | 0·017 | 0·012 |
| *Frozen* | | | | | | | | | | | |
| Single cream | 4 | 1 | 4·2 | 0·31 | 1·52 | 0·44 | 0·0037 | 0·57 | 2·89 | 0·025 | 0·082 |
| Whipping cream | 10 | 2 | 7·3 | 0·26 | 1·70 | 0·35 | 0·0026 | 0·33 | 1·89 | 0·015 | 0·084 |
| Sterilised canned cream | 13 | 5 | 0·3 | 0·16 | 1·57 | 0·22 | 0·0006 | 0·61 | 2·47 | 0·021 | 0·014 |
| Soured cultured cream | 8 | 4 | 1·8 | 0·31 | 1·67 | 0·39 | 0·0017 | 0·70 | 2·43 | 0·015 | 0·120 |

[a]Expressed as thiamin HCl, pyridoxal HCl and Ca-D-pantothenate, respectively.

### 2.3 Analytical methods

Total vitamin C was measured by the microfluorometric method essentially as described by Deustch and Weeks (1965). Vitamins of the B complex were analysed by microbiological methods as described by Scott *et al* (1984).

## 3 RESULTS AND DISCUSSION

The sample protocol was designed to give a single representative sample of the particular type of cream or ice cream, and allows comparison only between these types, not within types.

### 3.1 Creams (see Table 1)

The concentration of water-soluble vitamins in creams will reflect to some extent the level of fat and the overall effects of the type of heat processing. This was more marked for some vitamins than for others. The contents of B vitamins in fresh double cream (~48% fat) compared with fresh single cream (~19% fat) were thiamin, 61%; vitamin $B_6$, 58%; nicotinic acid, 56%; pantothenic acid, 67%; and biotin, 61%; averaging 60%. The figure for vitamin $B_{12}$ was 76%; there were negligible differences in riboflavin and folic acid levels.

The effect of different processing conditions was particularly marked for vitamin C in UHT and canned sterilised creams, for folic acid in UHT and canned sterilised creams, and for vitamin $B_{12}$ in canned sterilised creams. Compared with pasteurised single cream, levels in UHT single cream for thiamin, vitamin $B_6$ and vitamin $B_{12}$ were on average 20% lower. Differences in levels of riboflavin, nicotinic acid, pantothenic acid and biotin were negligible but folic acid was 60% lower. The level of vitamin $B_{12}$ in sterilised cream was >50% lower; folic acid and vitamin $B_{12}$ were 79% and 76% lower, respectively. Vitamin C was virtually completely destroyed in both UHT and sterilised creams. Similar differences in vitamin levels were also found in UHT and sterilised full cream cows' milk compared with pasteurised milk (Scott and Bishop 1986).

Levels of vitamins in soured cultured cream were generally similar to those of single fresh cream; the level of folic acid was double. Table 2 shows the concentration of vitamins found in single, double, and whipping cream and in sterilised canned cream, compared with the values calculated from fresh whole milk as given in Paul and Southgate (1978). Some differences are evident, particularly for riboflavin and folic acid in single, double, whipping and sterilised cream.

### 3.2 Ice creams and milk shakes (Table 3)

Concentrations of individual vitamins in all types of ice cream were generally similar (see also Paul and Southgate 1978). However, with the exception of thiamin, all values were higher than those given in Paul and Southgate (1978). (See Table 2.) Concentrations were similar to or slightly higher than those in pasteurised milk. The concentrations of vitamins in milk shakes were very similar to those in pasteurised milk.

**TABLE 2**

Comparison of Content of Water-soluble Vitamins in Creams and Ice Creams with Published Data ($\mu g\ g^{-1}$)

| *Samples* | *Source* | *Total vitamin C* | *Thiamin* | *Riboflavin* | *Vitamin $B_6$* | *Vitamin $B_{12}$* | *Nicotinic acid* | *Panto-thenic acid* | *Biotin* | *Folic acid* |
|---|---|---|---|---|---|---|---|---|---|---|
| Single cream | 1 | 8·9 | 0·36 | 1·67 | 0·48 | 0·0025 | 0·70 | 2·84 | 0·018 | 0·068 |
| | 2 | 12·0 | 0·30 | 1·20 | 0·30 | 0·0020 | 0·70 | 3·00 | 0·014 | 0·040 |
| Double cream | 1 | 6·1 | 0·22 | 1·57 | 0·28 | 0·0019 | 0·39 | 1·91 | 0·011 | 0·066 |
| | 2 | 8·0 | 0·20 | 0·80 | 0·20 | 0·0010 | 0·40 | 1·90 | 0·008 | 0·020 |
| Whipping cream | 1 | 7·4 | 0·24 | 1·65 | 0·37 | 0·0021 | 0·39 | 2·21 | 0·014 | 0·070 |
| | 2 | 9·0 | 0·20 | 0·90 | 0·20 | 0·0020 | 0·50 | 2·10 | 0·009 | 0·030 |
| Sterilised cream | 1 | 0·3 | 0·16 | 1·57 | 0·22 | 0·0006 | 0·61 | 2·47 | 0·021 | 0·014 |
| | 2 | trace | 0·10 | 1·00 | 0·10 | trace | 0·60 | 2·80 | 0·013 | trace |
| Dairy and non-dairy ice cream | 1 | 10·2 | 0·40 | 2·48 | 0·73 | 0·0037 | 1·37 | 4·00 | 0·027 | 0·080 |
| | 2 | 0 | 0·40 | 1·62 | 0·20 | trace | 1·00 | — | — | 0·020 |
| Pasteurised full cream cows' milk | 3 | 8·5 | 0·39 | 1·72 | 0·62 | 0·0037 | 0·85 | 3·38 | 0·021 | 0·055 |

Source 1: This survey; 2: Paul and Southgate 1978; 3: Scott and Bishop 1986 (mean summer and winter).

**TABLE 3**

Content of Water-soluble Vitamins in Ice Creams and Milk Shakes ($\mu g\ g^{-1}$)

| *Samples* | *No of samples* | *No of outlets from which samples obtained* | *Total vitamin C* | *Thiamin*[a] | *Riboflavin* | *Vitamin $B_6$*[a] | *Vitamin $B_{12}$* | *Nicotinic acid* | *Panto-thenic acid*[a] | *Biotin* | *Folic acid* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dairy vanilla ice cream | 17 | 6 | 9·0 | 0·41 | 2·49 | 0·79 | 0·0038 | 1·28 | 4·36 | 0·025 | 0·070 |
| Dairy flavoured ice cream | 17 | 5 | 10·6 | 0·38 | 2·60 | 0·70 | 0·0027 | 1·68 | 3·32 | 0·024 | 0·091 |
| Non-dairy vanilla ice cream | 11 | 5 | 12·4 | 0·42 | 2·41 | 0·72 | 0·0047 | 1·17 | 4·29 | 0·030 | 0·082 |
| Non-dairy flavoured ice cream | 14 | 4 | 8·7 | 0·40 | 2·40 | 0·71 | 0·0037 | 1·35 | 4·03 | 0·028 | 0·077 |
| Ice cream 'mix' (prepared) | 2 | 1 | 5·2 | 0·48 | 2·28 | 0·76 | 0·0033 | 1·13 | 4·39 | 0·031 | 0·063 |
| Milk shake (take-away) | 21 | 6 | 7·7 | 0·35 | 1·77 | 0·55 | 0·0034 | 0·85 | 3·08 | 0·019 | 0·040 |

[a]Expressed as thiamin HCl, pyridoxal HCl and Ca-D-pantothenate, respectively.

An indication of the reliability of the data presented here may be obtained from the assessment of analytical variation in the microbiological assay of vitamins of the B complex based on the repeated analysis of a reference milk sample over a period of 18 months (Scott *et al* 1984). The coefficient of variance based on the standard deviation for a single determination was 7%, ranging from 2·4% for folic acid to 13·4% for nicotinic acid.

## 4 GENERAL

Although in the past a number of important investigations have been carried out to assess the effects of handling, processing and storage on the nutrient content of milk and milk products, there are no published data representative of those available on the current or potential retail market.

During the period 1980 to 1985 the Ministry of Agriculture, Fisheries and Food in London commissioned a number of long-term studies to provide detailed nutritional analysis and compositional information on a wide range of milk products. These have included studies on bulk liquid milk (Scott *et al* 1984; Scott and Bishop 1984; Florence *et al* 1985); baby milk formula (Scott and Bishop 1985); retail milk and milk products (Scott and Bishop 1986); and the concluding studies reported in this and the preceding paper (Scott and Bishop 1988) on cheeses, creams, ice creams and milk shakes.

Recently nutrient data studies have become all the more important for a number of reasons, among which is the growing concern about the nutritional adequacy of the UK diet and the possible health risks associated with it, and the introduction of nutritional labelling.

## ACKNOWLEDGEMENTS

This study forms part of a survey on the nutrient content of milk and milk products commissioned by the Food Science Division of the Ministry of Agriculture, Fisheries and Food, London. The contribution to the initiation and detailed planning of this study made by Dr D H Buss, Dr Hazel Tyler and Miss Helen Crawley is gratefully acknowledged.

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